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From the Institute of Physiology, University of Marburg Germany

Regional Blood Flow in the Cerebral Cortex, Measured Simultaneously by Heat and Inert Gas Clearance

By

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Abstract

Betz, E, D H Ingvar, N A Lassen and F W Schmahl *Regional blood flow in the cerebral cortex, measured simultaneously by heat and inert gas clearance* Acta physiol scand 1966 *67* 1—9

[illegible]

Recently two techniques have been developed for measurements of regional blood flow in the cerebral cortex Both are based on recording the clearance by the blood of a freely diffusible indicator administered to the cortex i e *heat* as used by Kanzow 1961), Betz and Wullenweber (1962) and Schmahl and Betz (1964), and ⁸⁵*Krypton*, a radioactive inert gas, as used by Lassen and Ingvar (1961) and Ingvar and Lassen (1962) The heat clearance (heat-conductivity) method (Gibbs 1933, Hensel 1953/1954) permits a continuous measurement of the local cerebral blood flow, but the absolute value of the cortical perfusion rate cannot be measured The ⁸⁵Kr clearance method is a discontinuous procedure which requires a constant blood flow during measurement With this method, however, the blood flow is measured in ml g min

The present report concerns simultaneous measurements in dogs and cats of regional cortical blood flow with the two methods in an attempt to calibrate the heat conductivity element in absolute terms

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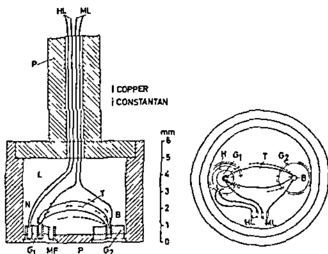


Fig 1 Vertical and horizontal section through the heat conductivity element used for recording regional blood flow on the cerebral cortex P Plexiglass MF measuring surface G_1 heated gold plate G_2 reference plate H heating coil T thermobattery HL heating leads ML measuring leads B bore hole for thermojunctions N groove for heating coil L air gap

Methods

1 Regional blood flow measurements by heat clearance

The measuring principle of heat conductivity elements for recording regional cerebral blood flow is the same as for the elements which have been used for recording local blood flow elsewhere in the body (Gibbs 1933 Grayson 1952 Hensel 1953/54 1956 1959 Perl 1962 Golenhofen Hensel and [illegible]

[illegible]

was kept below 3—5 g/cm²

Characteristics of the heat conductivity element

If a small part of a tissue is heated by a surface element with constant electric current (I) the heat clearance (λ) is given by the equation (Hensel 1961)

$$\lambda = \frac{K \cdot I^2}{\vartheta} - a$$

where K and a are empirically measured instrument constants and ϑ the continuously recorded temperature difference between the heated and the reference point The instrument constant K was measured on plane solid bodies of known heat conductivity by the procedure described by Golenhofen *et al* (1963)

In our experiments there was a co-heating of the reference gold plate when the other gold plate was heated On the surface of a 10 % gelatine gel (heat conductivity 12 5 10⁻⁴ cal cm sec °C) the reference junction was heated to 25 % of the temperature of the other plate (Fig 2 position G) On the living bra n [illegible] a model of a disc o [illegible] 200 μ in diameter [illegible] non perfused dead [illegible] position A) the heated point was heated more than in position B of Fig 2 when the heated plate was placed down-stream

¹ Manufacturer Hartmann & Braun Frankfurt M

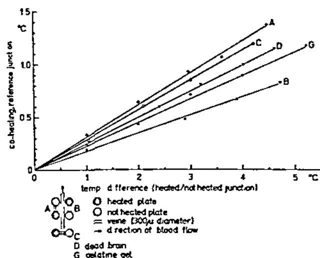


Fig 2 Diagram of co-heating of the reference junctions of a heat conductivity element (external diameter 8 mm) Diameter of the gold plates 2 mm Distance between the centers of the gold plates 5 7 mm (See text)



Fig 3 Cat Nembutal anesthesia Transparent model of a heat conductivity element placed on the cerebral cortex The diameter of the vessel between the two plates is 150 μ This position is suitable for measurements and corresponds to position C of Fig 2

under the heated gold plate by a micromanipulator One thermojunction was fitted upon the center of the measuring surface of the plate other thermocouples were placed at distances of 0 2—0 3 mm 0 5—0 6 mm 1 mm, and 2—3 mm from the center of the platelet inside the brain When plate G_1 was heated the decrease in tissue temperature with increasing vertical depth from the heater was found to be approximately exponential, varying slightly from experiment to experiment. Since the penetration depth of our element for blood flow measurements is about 1 5 mm in the tissue only changes within about 75 % of the temperature increment vertically below the heated platelet have essential influence on the blood flow measurements cf Golenhofen *et al* 1963

The differences in the co-heating and in the temperature fields caused by the non homogeneity of the local heat clearance can lead to relatively large variations in relation to local blood flow A calibration by artificial perfusion is therefore often used (Betz Braasch and Hensel 1961 Graf Golenhofen and Hensel 1959 Betz Gaver and Weber 1964

Linzell (1953 measured the heat transport from a heated thermocouple in perfused simple narrow tubes The I² required to keep the temperature of the heated thermojunction at 1° C above the un-

heated one was not linearly proportional to the blood flow per time ($\bar{f}$), but was, in the middle flow

(Betz and Hensel 1962 Betz *et al* 1964) The results obtained by artificial perfusion do not permit any conclusions on the extent to which the total blood flow represents the local blood flow Thus it is necessary to calibrate each position of a heat conductivity element by a method which gives absolute values for the regional blood flow

2 Regional blood flow measurement by ^{85}Kr clearance

The technique used was a modification of the intra arterial ^{85}Kr injection method of Ingvar and Lassen (1962)

arterially the build up and subsequent regional cortical clearance were followed by the small G M tube for 10–20 min This technique is simpler than the prolonged infusion method and it is also more economical with respect to isotope utilization It is probably also somewhat more accurate, since the calculation is based on measuring the height and the area of the clearance curve parameters which are easier to measure than the initial tangent

A brief description of the rapid injection technique will be given below with special regard to the amount of tissue measured from

^{85}Kr emits β particles of a maximal energy of 0 7 mev in 99 6 % of the disintegrations and γ radiation of an energy of 0 5 mev in the remaining 0 4 % Using a GM tube as detector, the role of this low incidence γ radiation becomes quite negligible, since these detectors have a very low sensitivity to such radiation

The isotope is obtained at a specific activity of 1 0 Curie per 13 6 ml of gas (at atmospheric pressure) It is dissolved in a 100 ml syringe in sterile saline which is used as stock solution Of this solution 0 2 to 1 0 ml was used per measurement The ^{85}Kr saline was injected during 1–2 sec via a polyethylene catheter into the arterial inflow to the brain

The dead space of the catheter was not flushed, in order to obtain defined conditions for the rapid

(5 μ thick) placed on the cortical surface was continuously renewed by a gentle stream of air Care was taken to remove any blood or cerebrospinal fluid close to the counting area Such stagnant fluid layers might contain small amounts of isotope and thus distort the clearance curve

The influence of the superficial vessels on the ^{85}Kr clearance was found to be negligible as no significant changes in calculated flow were observed under steady state conditions when the probe was moved from one site on a gyrus to an adjacent one An artifact was seen only when the probe was erroneously placed over a sulcus with arteries carrying ^{85}Kr to other regions In that case an excessively high initial peak was seen on the curve corresponding to the rapid transit of the indicator bolus destined to other areas (cf Haggendal *et al* 1965) By always placing the G M tube over a gyrus this artifact was avoided

The lack of sensitivity of the ^{85}Kr method to the minute vascular anatomy of the cortex is probably

the walls of larger vessels

The range of the β radiation of ^{85}Kr in tissue is 1 5 mm this is the layer from which 95 % of the recorded emission emanates A layer of similar thickness is measured by the heat clearance method and in both methods the efficiency falls off almost exponentially with increasing depth

The counting rate was not allowed to exceed 15 000 cpm a rate at which the coincidence loss of the G M tube starts to become significant (dead time 80 μ sec) The impulses were integrated using a ratemeter with a time constant of 1 0 sec coupled to a linear potentiometer recorder with a full scale response of 1 0 sec The curve was followed until it practically reached the background level (10 to 20 min) The total number of impulses recorded over the same interval was also observed on a scaler This total count (minus background represents the area A under the clearance curve (Fig 4)

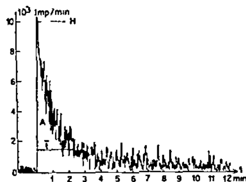


Fig 4 Cat, Nembutal anesthesia Original record Clearance curve from the cerebral cortex following injection of ⁸⁵Kr, dissolved in saline, into the carotid artery H initial height of the curve = total amount of indicator seen by the G-M tube, A Area t measuring time, t̄ mean transit time (See text.)

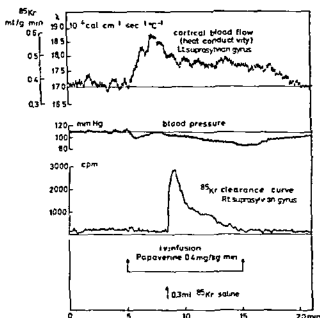


Fig 5 Cat Nembutal anesthesia Records of 1) heat clearance on the frontal part of the left suprasylvian gyrus 2) mean blood pressure, 3) ⁸⁵Kr clearance on a symmetrical point of the right suprasylvian gyrus Injection of ⁸⁵Kr saline into the right lingual artery Note inconstant blood flow during the first minutes following the start of the papaverine infusion and at the end of the recording

Calculation of blood flow from the ⁸⁵Kr clearance observed

The observed transit of the ⁸⁵Kr bolus through the cortical area recorded from enables one to calculate the average tissue blood flow provided three assumptions can be made Firstly that the blood flow is reasonably constant during the clearance period This was checked in all cases by the heat-conductivity method and only observations where this was the case were regarded as valid (Fig 5

Sec ... obably true since the ... h as water and lipid ... An average value of ... out disregarding the ... 1962

Thirdly that the maximal height of the clearance curve represents the counting rate of all the indicator which will be cleared by the region In order to meet this requirement the injection must be rapid and a short ratemeter constant must be used Then at the time of maximum concentration essentially all of the isotope has arrived at the area and has been recorded before any appreciable clearance from the area has taken place

Absence of indicator recirculation is also necessary, if this third assumption is to be fulfilled This was checked by the absence of measurable increase in the cortical counting rate following an i v injection of a standard dose of ⁸⁵Kr saline

Based on these assumption, it can be shown that the area, A, under the clearance curve until infinity, divided by its maximal height, H, is the mean transit time, $\bar{t}$, in minutes of ^{85}Kr through the area (Zierler 1965, Hoedt Rasmussen, Sveinsdottir and Lassen 1966)

$$\bar{t} = A/H \text{ minutes} \qquad (1)$$

Th f ndamental ...

elei

am

of time, t, they take to pass through the field (Fig 4)

Thus the mean of all the t values, $\bar{t}$, can be calculated directly, if one regards t as a function of the height

$$\bar{t} = \frac{\int_0^H t(h)dh}{\int_0^H dh} = \frac{A}{H}$$

The mean transit time of ^{85}Kr in the region studied must equal the ratio of its distribution space V and the total blood flow F

$$\bar{t} = V/F \qquad (2)$$

Rearranging (2) and defining the perfusion rate, f, as the blood flow per unit weight of tissue (f = F/W, W being the number of g of tissue recorded from), one obtains

$$f = (V/W) \quad 1/\bar{t} \text{ [ml/g min]} \qquad (3)$$

Inserting (1) and noting that the distribution space for ^{85}Kr per g (V/W) is by definition α'[ml/g], equation (3) becomes

$$f \simeq \alpha' \quad (H - H_{(10)})/A_{(10)} \text{ [ml/g min]} \qquad (5)$$

mation of about 10 per cent Applied to the cortex alone, a tissue which is more homogeneous and has a higher perfusion rate than the total brain this overestimation is less pronounced, probably only a few per cent at a flow level of about 0 80 ml/g min This estimate is supported by calculations where mono-exponential extrapolation of the cortical clearance curves beyond the actual observation period was made in order to estimate the total area A

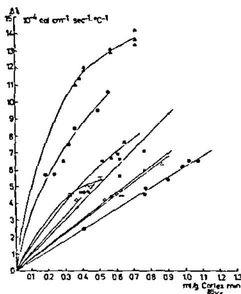
Experimental procedures

The cortical heat and ^{85}Kr clearance was measured simultaneously in 10 cats and 6 dogs The animals were anesthetized initially with 25 mg/kg Pentobarbital i v , and maintained on Nembutal In 5 of the dog experiments simultaneous measurements of the cortical oxygen uptake the EEG, and the cortical tissue pO_2 were made The results of these measurements have been published separately (Lubbers *et al* 1964)

The disc with the heat conductivity element and the G M tube were placed either on the exposed surface of the supra sylvian gyrus of one hemisphere or on symmetric areas on the same gyrus on both sides The isotope was injected through a thin polyethylene cannula which was mounted in the central end of the cut lingual artery on the side of the hemisphere to be studied with the ^{85}Kr-method The

... sinus nerve was cut and the injected volume decreased, the blood flow reactions were negligible In dogs such reactions were not seen when the injected volume was kept below 0 3 ml

Increased rates of cortical blood flow were obtained by i v infusions of nor-epinephrine, or papaverine The increase was however often so variable that steady-state conditions were not achieved The only isotope clearance values used for calculation were those obtained when the heat clearance varied less than 0 4 10 cal cm^{-1} sec^{-1} °C^{-1} (Fig 5) It was found easier to keep the cortical blood flow at a constantly increased rate when applying CO_2 Concentrations of 3 %, 5 % and 7 % in air were used



correlations were obtained on regions with only small vessels (diameter below 100 μ) The other curves were obtained from areas with small vessels as well as some vessels larger than 100 μ

On the other hand the blood flow did not change much during slow bleeding until a blood pressure level of 80—70 mm Hg was reached After lowering the blood pressure below about 70 mm Hg the blood flow decreased but in many cases it did not remain at a constant level for the needed time In the final states of hypotension however conditions suitable for calibration were usually achieved

Results

The heat clearance of the cortex always shows more or less pronounced variations They represent variations in perfusion due to such factors as spontaneous changes of blood pressure, changes in respiration, and independent periodic blood flow changes of a low frequency (Fig 5) A primary point of the present study was to establish whether a relatively stable continuous record obtained from the heat clearance element corresponded to stable ^{85}Kr values From 24 determinations in 6 different experiments, selected from situations when the variations in heat clearance were minimal (see above) during ^{85}Kr clearance, coefficients of variation of 5 1 % for ^{85}Kr values and of 4 7 % for λ values were obtained

The second point was the relationship between heat clearance and ^{85}Kr clearance at different flow rates The correlations obtained are seen in Fig 6 Nearly linear relations were found when the measuring area for heat clearance had no vessels with a diameter exceeding 100—150 μ It is obvious however from Fig 6 that the slopes of the curves obtained varied from one experiment to another

Discussion

In the present experiments the two methods were used on symmetrical cortical areas It is unlikely that the blood flow in such areas differs more than a few per cent This is apparent from the marked homogeneity of cortical blood flow from area to area during anesthesia as revealed by the autoradiographic studies of lo et al

(1961) In no instance were right — left differences found in corresponding areas This is also supported by simultaneous heat clearance studies over symmetrical cortical areas (Schmahl and Betz 1964, Betz 1965)

On this basis, and in view of the remarkable similarity of the thickness (1 5 mm) of the corresponding cortical layers measured from, and in depth sensitivity (exponential decrease), it is concluded that the two methods measure essentially from the same tissue In previous attempts at calibrating heat conductivity probes, this method for local flow measurement has been compared with total flow measurements (Grayson 1952, Betz and Hensel 1962) In such studies any lack of strict proportionality between local and total flow constitutes an uncontrollable source of variation This fundamental difficulty is circumvented by the present approach

The ^{85}Kr method presupposes a steady state of blood flow during the clearance period This important assumption was checked by continuous measurements of the heat clearance with the heat conductivity element Experiments, in which variations of regional blood flow in the ipsilateral hemisphere were caused by the isotope injection were discarded Thus there was no "injection artifact" resulting from the isotope injection Moreover, a reasonably constant blood flow during the entire ^{85}Kr clearance period (10 to 20 min) could be assured by simply ignoring those studies in which heat clearance variations showed that this was not the case Having thus satisfied the steady state condition, there seems to be little reason to doubt the correctness of the flow values obtained with ^{85}Kr On this basis it is considered justified to conceive of the present correlative studies as a calibration of the heat conductivity method by the ^{85}Kr method, a calibration which, as just mentioned, is theoretically impossible by total brain blood flow measurements It is of interest, however, to note in this context that the relation between $\Delta\lambda$ and ^{85}Kr clearance as found in the present study is, in all qualitative respects, similar to that found by total cerebral blood flow measurements (Perl 1962, Grayson 1952) Apparently, under the conditions studied, total flow and cortical flow have changed in a parallel manner hence obviating, to some extent at least, the criticisms here raised

In contrast to ^{85}Kr clearance within the cerebral cortex, heat clearance depends as mentioned quite markedly on the exact location of the thermoprobe in relation to larger pial vessels Thus there cannot be any question of the qualitative nature of the method, a fact that has been repeatedly stressed (cf Golenhofen *et al* 1963) On the other hand, at any given location, the heat clearance responds very consistently to stimuli (e g CO_2) that can be considered to cause a given, constant change in local blood flow A constant responsivity of the heat probe is maintained even over many months in chronic experiments (Betz 1965) This constancy of response is of considerable importance as it suggests that for any given location of the probe, calibration of it by means of the ^{85}Kr method need only be made once In this way the simple and untraumatic heat-conductivity method may be converted into a quantitative method for continuous measurement of local blood flow

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The Alveolar-Arterial Oxygen Difference: Its Size and Components in Normal Man

By

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Abstract

MELLEMGAARD, K *The alveolar-arterial oxygen difference Its size and components in normal man* Acta physiol scand 1966 67 10—20

[illegible]

breathing was used for measurement of the total venoarterial shunt, whereas analysis of the concentration of tritium (hydrogen) in arterial blood after intravenous injection of this gas in physical solution [illegible]

perfusion ratio in the lungs

Since the introduction of reliable methods for O_2 tension measurements in blood, a considerable amount of data on the normal arterial O_2 tension and alveolar-arterial A-a O_2 difference has been reported In most studies, however, a greater part of the observations has been made on young normal subjects, so that there is a lack of data concerning the older age groups Only recently have systematic investigations of age variations in these parameters been carried out

The present paper describes the results of determination of arterial O_2 tension and A-a O_2 difference in a normal human material with a large age spectrum It includes also an attempt to separate the components of the A-a O_2 difference in normal human subjects For this purpose two different techniques were employed Determination of venoarterial shunt by measurement of the A-a O_2 tension difference during O_2 breathing (Berggren 1942), and determination of right-to-left shunt through the central circulation with an inert gas technique (Lassen, Mellemgaard and Georg 1961).

Material

... on an approximately even distribution through the

...

results

Experimental procedure

The subjects walked to the laboratory, where they were seated comfortably in a deck-chair with about

...

repeated after 30 and 45 min

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Methods

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polarographic technique for full blood based on the dropping mercury electrode The error of the method was estimated from a series of tonometer experiments repeated at intervals The standard

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determination relative to the second calibration In actual measurements 3 separately drawn blood samples were analyzed immediately and the results compared with a calibration curve from simultaneously drawn blood equilibrated in rocking ball tonometers (Laue 1951) with gas mixtures with P_{CO_2} about 40 mm Hg and P_{O_2} somewhat higher and somewhat lower than that of the blood sample Analysis and equilibration were made in the same thermostated water bath at 37.5° ± 0.1 The results are given as average values of three measurements

...

ing PO_2 immediately after sampling, or — in a few cases where this was not possible — by storing in ice water. Since measurements of equilibrated samples took about the same time as measurements of actual samples, and since there was no significant difference between the means of the first and last analyzed samples, a correction for O_2 consumption of the blood was not applied. A measuring temperature of 37.5° was chosen as a reasonable value of alveolar capillary temperature. This is equal to the average alveolar capillary temperature in normal man given by Edwards, Velasquez and Farhi (1963).

Analysis of tritium (T_2) and tritiated water (THO) was carried out as described by Lassen *et al.* (1961). The sample is divided into two, one being analyzed for THO after evacuation of T_2 gas. The other portion is analyzed for THO after conversion of the T_2 gas to THO by combustion, the predetermined THO being subtracted as a blank. THO was measured by the methane proportional counting method described by Robinson (1955). In 8 sets in which the same sample was analyzed 10 times, the coefficient of variation did not exceed 1.5 per cent.

Terminology and calculations

The generally accepted symbols and abbreviations (Pappenheimer *et al.* 1950) are employed with a few modifications. The suffix (ox) is used to indicate values obtained during O_2 breathing. V_D is used without suffix to indicate physiological dead space. There is no generally accepted symbol for *alveolar-arterial oxygen tension difference*, for which the symbol $P_{(A-a)O_2}$ is chosen here. *The arterial oxygen deficit*, defined as the difference between the O_2 content of blood in full equilibration with the mean alveolar air and the actual O_2 content of the arterial blood (Berggren 1942, Asmussen and Nielsen 1960) is correspondingly symbolized by $C_{(A-a)O_2}$. It is calculated from P_{AO_2}, P_{aO_2}, the oxygen capacity estimated from the hemoglobin concentration, the standard oxyhemoglobin dissociation curve of Dill and Forbes (Severinghaus 1958) and the solubility of O_2 in blood (Sendroy, Dillon and van Slyke 1934).

The mean alveolar oxygen tension P_{AO_2} is calculated from the alveolar air equation, substituting P_{aCO_2} for P_{ACO_2}. This calculation will not be strictly valid in the presence of an A-a P_{CO_2} difference due to ventilation perfusion inequalities or venoarterial shunt. Furthermore it appears that differences in diffusion velocities in the gas phase of the lungs may be of importance, since such differences can be demonstrated even in normal subjects (Georg *et al.* 1965). For these reasons the calculated P_{AO_2} and $P_{(A-a)O_2}$ must be considered a *minimum value*, although the error is probably small in normal conditions.

The mean alveolar oxygen tension during oxygen breathing is calculated from

$$P_{AO_2\,ox} = 0.99\,(P_B - 47) - P_{aCO_2}$$

thereby allowing for the small inert gas content in pure O_2 (0.3–0.5 %) and a small contribution to alveolar air of N_2 from tissue washout, which in ideal circumstances (evenly distributed ventilation/perfusion ratio) should result in an alveolar nitrogen fraction of about 0.3 % after 15 min O_2 breathing (Lundin 1953–54).

The physiological dead space V_D is calculated according to the Bohr equation substituting P_{aCO_2} for P_{ACO_2}. Corrections are made for the dead space of the directional valves (10 ml) and mouthpiece (20 ml).

The physiological shunt Q_s (venous admixture) is used to indicate the fractional perfusion through nonventilated alveoli and "anatomical" shunts which would account for the observed arterial O_2 deficit during respiration of atmospheric air. It is calculated from the shunt equation

$$\frac{Q_s}{Q} = \frac{C_{cO_2} - C_{aO_2}}{C_{cO_2} - C_{\bar{v}O_2}} = \frac{C_{(A-a)O_2}}{C_{(A-a)O_2} + C_{(a-\bar{v})O_2}}$$

assuming full equilibration between end-capillary oxygen tension and mean alveolar oxygen tension. The *venoarterial shunt* Q_{va} ("anatomical" shunt) is calculated in similar way from the data obtained during oxygen breathing

$$\frac{Q_{va}}{Q} = \frac{C_{(A-a)O_2\,ox}}{C_{(A-a)O_2\,ox} + C_{(a-\bar{v})O_2}}$$

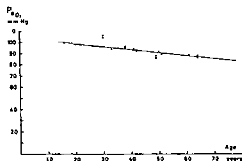
The arteriovenous oxygen difference $C_{(a-\bar{v})O_2}$ was not measured. An average value of 45 ml per liter was assumed. The use of this fixed value would give rise to large errors if applied to individual results. The shunt calculations are therefore carried out only for average values of arterial O_2 deficit.

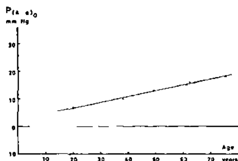
The *right-to-left shunt* in the central circulation Q_{rl} is estimated from the calculated "recovery" (R_{T_2}) of tritium from arterial blood sampled during the first circulation, i.e. the ratio of tritium to tritiated water in the arterial blood (r_a) relative to the corresponding ratio (r_i) in an anaerobically produced standard solution of the injectate in blank blood from the subject:

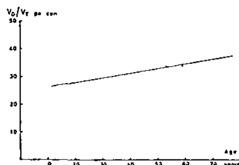
$$R_{T_2} = \frac{r_a}{r_i} = \frac{C_aT_2 / C_aTHO}{C_iT_2 / C_iTHO}$$

r_a is a measure of the ratio that would have been found in the arterial sample if no loss of T_2 to the gas phase had occurred. For reasons discussed below R_{T_2} represents a maximal value of Q_{rl}.

Standard statistical methods (Snedecor 1956) were used in the treatment of the results.







shown

Results

Table I shows the results A) divided into 6 age groups, B) divided into 2 equally sized age groups 15—40 and 41—75 years, and C) as average values for the total material. Individual values of arterial O_2 tension, A-a O_2 tension difference, and physiological dead space ratio are given in Fig. 1—3. All results during O_2 breathing were obtained after 15 min. In 9 subjects O_2 breathing was continued for 45 min. The average values of $P_{aO_2(ox)}$ after 15, 30, and 45 min: 620.2 mm Hg, 626.3 mm Hg, and 615.0 mm Hg were not significantly different.

Age variations. While P_{aCO_2} and P_{AO_2} showed no significant variation with age, the regression of P_{aO_2} on age was highly significant (Fig. 1). The regression was $P_{aO_2} = 104.2 — 0.27$ age. Addition of the equally significant regression of P_{aO_2} on P_{AO_2} ($p < 0.001$) results in the multiple regression $P_{aO_2} = 0.58\ P_{AO_2} — 0.24$ age

TABLE I

Age group		n	Age years	Respiration in atmospheric air P_{aCO2} mm Hg	P_{AO2} mm Hg	P_{aO2} mm Hg	$P_{(A-a)O2}$ mm Hg	$C_{(A-a)O2}$ ml/l
15—20	Mean	12	17 8	37 7	103 9	98 9	5 0	0 92
	s d		1 6	1 8	4 0	5 1	5 5	0 99
21—30	Mean	16	25 8	37 9	106 0	97 8	8 3	1 51
	s d		3 0	3 6	6 4	6 6	6 2	1 14
31—40	Mean	12	35 5	38 4	104 2	93 1	11 1	2 20
	s d		2 5	2 5	3 5	5 6	6 3	1 37
41—50	Mean	16	46 9	38 7	104 3	92 6	11 7	2 41
	s d		2 8	1 7	3 8	9 1	10 2	2 00
51—60	Mean	12	54 6	39 0	102 2	87 9	14 3	3 03
	s d		2 8	3 5	6 1	6 9	5 9	1 28
61—75	Mean	12	66 3	39 0	103 0	86 7	16 3	3 49
	s d		5 0	2 3	6 7	8 3	3 8	1 29
15—40	Mean	40	26 3	38 0	104 8	96 7	8 1	1 54
	s d		7 4	2 8	5 0	6 2	6 4	1 25
	s e of mean		1 2	0 4	0 8	1 0	1 0	0 20
41—75	Mean	40	55 1	38 9	103 3	89 4	13 9	2 9?
	s d		8 9	2 9	5 3	8 0	6 9	1 64
	s e of mean		1 4	0 5	0 8	1 3	1 1	0 26
$\bar{x}_{>40} - \bar{x}_{<40}$				0 9	1 5	7 3	5 8	1 38
$s(\bar{x}_{>40} - \bar{x}_{<40})$				0 63	1 15	1 60	1 48	0 33
P				0 1 < P 0 2	0 1 < P < 0 2	< 0 001	< 0 001	< 0 001
15—75	Mean	80	40 7	38 4	104 0	93 1	11 0	2.23
	s d		16 6	2 9	5 2	8 0	7 1	1 61
	s e of mean		1 9	0 3	0 6	0 9	0 8	0 18

+ 42 7 This was shown by analysis of variance to give a slight improvement of the estimate of P_{aO_2} s d 6 0 mm Hg)

The regression of $P_{A-a\,O_2}$ on age was also highly significant (Fig 2) The ratio of physiological dead space to tidal volume showed large variations within the age groups There was however a significant increase of V_D/V_T with increasing age (Fig 3) Since the A a O_2 difference and the dead space ratio can both be taken to indicate the degree of ventilation perfusion inequalities the relationship between these two parameters was investigated The correlation between $P_{(A-a)O_2}$ and V_D/V_T was not significant (p > 0 05) whereas the correlation between $C_{(A-a)O_2}$ and V_D/V_T was slightly better (0 01 < p < 0 05)

The regression on age of $P_{aO_2(ox)}$ (0 001 < p < 0 005) and $P_{A-a\,O_2(ox)}$ (0 001 <

	Respiration in pure oxygen						
$\dot{V}_D/\dot{V}_T$ per cent	$P_{aCO_2(ox)}$ mm Hg	$P_{AO_2(ox)}$ mm Hg	$P_{aO_2(ox)}$ mm Hg	$P_{(A-a)O_2}$ (ox) mm Hg	$C_{(A-a)O_2}$ (ox) ml/l	$C_{(A-a)O_2}$ $C_{(A-a)O_2}$ (ox) ml/l	R_{T2} (n =)
25.3	37.5	662.2	635.6	26.8	0.83	0.09	0.99 (3)
4.8	3.7	10.5	23.7	20.8	0.65	0.81	0.76
28.5	38.9	666.8	633.2	33.6	1.04	0.46	0.94 (10)
7.5	4.0	9.4	34.3	33.2	1.03	0.71	0.47
33.6	38.8	668.9	615.5	53.4	1.66	0.54	1.11 (10)
6.7	2.5	7.5	19.6	36.1	1.12	0.87	0.36
32.9	38.1	670.6	616.4	54.2	1.68	0.73	1.23 (11)
8.1	2.9	7.6	39.4	38.9	0.92	1.70	0.68
36.3	40.6	662.0	596.9	65.1	2.02	1.01	1.49 (8)
6.4	3.0	9.7	42.8	39.3	1.29	1.11	0.80
32.0	39.0	664.8	605.2	59.6	1.85	1.64	1.13 (7)
6.0	2.7	12.0	38.7	35.1	1.09	1.04	0.46
29.1	38.5	666.1	628.6	37.5	1.16	0.38	1.02 (23)
7.2	3.5	9.4	28.2	37.1	1.00	0.79	0.45
1.1	0.6	1.5	4.5	5.1	0.16	0.12	0.09
33.7	39.1	666.3	607.2	59.1	1.83	1.09	1.28 (26)
7.1	3.0	10.1	40.1	37.2	1.05	1.38	0.66
1.1	0.5	1.6	6.3	5.9	0.17	0.22	0.13
4.6	0.6	0.2	21.4	21.6	0.67	0.71	0.26
1.6	0.73	2.19	7.7	7.8	0.23	0.25	0.16
<0.01	0.4<P <0.5	0.9<P <0.95	0.005<P <0.01	0.005<P <0.01	0.001<P <0.005	0.005<P <0.01	0.1<P <0.2
31.4	38.8	666.2	617.9	48.3	1.50	0.73	1.16 (49)
7.5	3.3	9.7	36.1	36.2	1.07	1.17	0.58
0.8	0.4	1.1	4.0	4.0	0.12	0.13	0.08

$p < 0.005$) were both significant. The difference between the arterial O_2 deficit during air breathing (C_{A-aO_2}) and that during O_2 breathing ($C_{A-aO_2,ox}$), which is an estimate of the minimal contribution from ventilation perfusion inequalities (see discussion), was also significantly correlated to age ($p < 0.001$).

Results expressed in terms of intrapulmonary shunt. The physiological shunt Q_s calculated from the average value of $C_{A-aO_2} = 2.23$ ml/l and an assumed a-v O_2 difference of 45 ml/l is 4.7 per cent. The venoarterial shunt Q_{va}, calculated from the average value of $C_{A-aO_2,ox} = 1.50$ ml/l and the same assumed avd is 3.2 per cent. If the two parameters were calculated for individual values of arterial O_2 deficit, they would show a significant rise with increasing age. This holds true even if allowance is made for the slight increase of avd observed with increasing age (Strandell 1964).

The right-to-left shunt Q_{rl} was determined in 49 of the 80 normal persons Apart from the small number in the youngest age group, the smaller material seems representative of the total material The regressions on age of P_{aO_2} ($p < 0.001$), $P_{(A-a)O_2}$ ($p < 0.005$) and $C_{(A-a)O_2}$ ($p < 0.001$) were significant However, the regressions of $P_{aO_2(ox)}$ ($0.05 < p < 0.1$) and $P_{(A-a)O_2(ox)}$ on age ($0.05 < p < 0.1$) were not significant in the smaller material

The mean recovery of tritium (R_{T_2}) is 1 16 per cent Regression calculation shows no significant correlation between R_{T_2} and age ($p = 0.1$), nor between R_{T_2} and P_{aO_2}, $C_{(A-a)O_2}$, $P_{aO_2(ox)}$ or $C_{(A-a)O_2(ox)}$

Discussion

The average value and range of arterial O_2 tension reported here is in agreement with most of the published larger series investigated with the present method (Bartels and Rodewald 1952) and other polarographic techniques (Cosby *et al* 1962, Raine and Bishop 1963, Ulmer and Reichel 1963, Cole and Bishop 1963, Worth, Muysers and Siehoff 1963)

Microtonometric measurements have given higher results in some studies (Suskind *et al* 1950) lower results in some (Asmussen and Nielsen 1960) and results comparable to the present in some (Wessel Aas 1958)

Age variation in A-a O_2 difference and physiological dead space A tendency to lower P_{aO_2} and higher $P_{(A-a)O_2}$ in the older age groups has been observed in some studies (Filley, Gregoire and Wright 1954, Wessel Aas 1958, Aksnes 1962) Recent investigations have shown age variations similar to those reported here P_{AO_2} has generally been unchanged with age, whereas approximately linear decreases of P_{aO_2} with age have been found in materials of 49 (Raine and Bishop 1963), 73 (Worth *et al* 1963) and 88 (Ulmer and Reichel 1963) normal subjects The slope of P_{aO_2}/age in these studies was about 0 24, 0 29 and 0 22 mm Hg/year respectively, compared with 0 27 mm Hg/year in the present material A steeper slope (about 0 5 mm Hg/year) was found by P_{O_2} determinations on "arterilized" ear lobe blood in a large material by Loew and Thews (1962)

These studies thus show, that there is a considerable variation with age of arterial O_2 tension and A a O_2 difference, so that definition of normal values without regard to age is hardly justified If the P_{aO_2}/age regression minus two times s d is accepted as a lower limit of the normal arterial O_2 tension, the minimal values will be approximately 85 mm Hg at 20, 80 mm Hg at 40 and 75 mm Hg at 60 years of age Correspondingly defined from $P_{(A-a)O_2}$/age-regression the upper normal limit of A-a difference will be 19 mm Hg at 20, 24 mm Hg at 40 and 28 mm Hg at 60 years of age

The physiological dead space found in the present study is on the average a little less than one third of the tidal volume This is in agreement with the majority of published results on seated normal subjects in resting conditions (Filley, Gregoire and Wright 1954, Asmussen and Nielsen 1956, Aksnes 1962, Larson and Severinghaus 1962, Raine and Bishop 1963, Cole and Bishop 1963) Only a few of these

studies include data from higher age groups In the material of Filley *et al* there is a tendency to increase of V_D/V_T with increasing age The results of Aksnes (1962) show no significant increase in V_D with age, but the studies of Raine and Bishop (1963) and those of Cole and Bishop (1963) demonstrate, like those presented here, a slight but statistically significant increase of V_D/V_T with age

Composition of A a O_2 difference

The deficit in equilibration between mean alveolar and arterial O_2 tensions is currently ascribed to 3 factors diffusion gradient, venoarterial shunt, and inequalities in the distribution of ventilation versus perfusion Theoretical considerations as well as experimental evidence (Staub 1963) indicate that there is no measurable gradient between oxygen tensions of gas and blood leaving the individual alveoli in normal subjects breathing atmospheric or O_2 enriched air There remains, however, the possibility, that differences in the distribution of diffusion capacity and perfusion to different lung sections could give rise to gradients between mean alveolar and end capillary O_2 tensions (Visser and Maas 1959 Piiper 1961) With the methods presently available the effects of such variations could not be distinguished from the effects of differences in distribution of ventilation and perfusion The present study gives no information concerning diffusion gradients In the following discussion it is therefore assumed, that there is complete equilibration between mean alveolar and end capillary O_2 tensions

The separation of shunt component and distribution component of $P_{(A-a)O_2}$ is in the present material attempted through direct measurement of the former This is done by two methods measuring different parameters The oxygen method gives an estimate of the total venoarterial shunt (Q_{va}) whereas the inert gas method measures only the fraction of venoarterial shunt originating from mixed venous blood in the right side of the heart (Q_{rl}) If both methods were equally valid the difference between Q_{va} and Q_{rl} would give a reliable estimate of the contribution to venoarterial shunt from the systemic circulation i e bronchial and thebesian drainage to the left side of the heart However, none of the methods can be expected to give the exactly correct result The most important sources of systematic error of the oxygen method are insufficient washout of nitrogen from the lungs and development of absorption atelectases during the procedure Both errors tend to result in overestimation of Q_{va} although probably only to a slight degree in normal subjects The inert gas method overestimates Q_{rl} because the arterial blood sampled during the first circulation after intravenous injection contains a small contribution of the gas from pulmonary capillary blood in equilibrium with alveolar air The size of this fraction depends on the partition coefficient λ_T 0 017 Mellemgaard, Lassen and Georg 1962)) and the relative volumes of blood and gas that reaches diffusion equilibrium in the lungs In studies on normal human subjects the relative contributions of shunt and "alveolar back pressure has been separated by simultaneous application of two inert gases (I and ⁸⁵ Kr) with different solubilities (Mellemgaard *et al* 1962) It was found that of an average R_{T_x} of 1 2 per cent appro[illegible]

per cent could be ascribed to the effect of right to left shunt. Accordingly any individaul R_{T_2} represent a maximal value of right-to-left shunt. For these reasons the interpretation of the present results requires some caution.

1. *Right to left shunt.* The average R_{T_2} of 1.16 per cent represent a maximal value of the mean right-to left shunt, since it includes a contribution from "alveolar back pressure" of at least 0.5 per cent. The conclusion is that the "true" right to left shunt in normal lungs is very small. This is in agreement with results obtained with other methods by Cole and Bishop (1963) and Lenfant (1963 and 1964). If an a-v O_2 difference of 45 ml/l is accepted, a pulmonary artery to vein shunt of 1/3 per cent contributes with an arterial oxygen deficit of 0.15 ml/l, which is 0.15/2.23 = 7 per cent of the mean total $C_{(A-a)O_2}$ or 0.15/1.50 = 10 per cent of the mean $C_{(A-a)O_2}$ during oxygen breathing.

2. *Contribution from bronchial and thebesian veins.* This is represented by the difference between $C_{(A-a)O_2(ox)}$ and the small $C_{(A-a)O_2}$ due to right to-left shunt. Using the mean values this gives a $C_{(A-a)O_2}$ of 1.50—0.15 ml/l, which is 90 per cent of $C_{(A-a)O(ox)}$ and 60 per cent of the total $C_{(A-a)O_2}$. Although the validity of $C_{(A-a)O_2(ox)}$ as a quantitative measure of venoarterial shunt is somewhat dubious, this suggests that the greater part of $C_{(A-a)O_2(ox)}$ is caused by bronchial and thebesian veins. On the other hand, the conclusion that more than half of the total $C_{(A-a)O_2}$ is caused by this contribution is probably an overestimation. The significant age variation of $C_{(A-a)O_2(ox)}$ in contrast to the constancy of R_{T_2} supports this view and suggests that $C_{(A-a)O_2(ox)}$ includes contributions from the distribution component, although age variations in thebesian and bronchial contribution can not be excluded.

Direct estimation of the contribution from thebesian veins to venoarterial shunt has been attempted by comparing P_{O_2} of blood from left atrium and systemic arteries. Bjork, Malmstrom and Uggla (1954) found large tension differences (average 7.5 mm Hg, range — 1.5 to 14) in patients with mitral stenosis, whereas Bartels *et al.* (1956) found an average difference of 2.9 mm Hg (range 0—10) in patients with mostly non cardiac diseases. The principal problem in such investigations is probably the efficiency of mixing in the left atrium.

Estimations of the contribution to venoarterial shunt from the bronchial circulation in normal human subjects are lacking. Total bronchial to pulmonary flow has been measured with dye dilution methods by sampling from the left atrium (Cudkowicz *et al.* 1960) or by comparison of the simultaneous output of the left and right heart (Fritts *et al.* 1961). The results obtained have necessarily shown wide variations. An average difference between left and right heart output of 0.9 per cent was reported by Fritts *et al.* 1961. Since it is not known to what extent this flow occurs through precapillary or postcapillary anastomoses in the lung, the result can in this connection serve only as a maximal value for the contribution of the bronchial circulation to the venoarterial shunt.

3. *Distribution component.* The minimal value of this contribution is $C_{(A-a)O_2}$ — $C_{(A-a)O_2(ox)}$ or, using the mean values, 2.23—1.50 ml/l or 1/3 of $C_{(A-a)O_2}$. Since a

considerable fraction of $C_{(A-a\ O\ (ox}$ is probably also caused by V_A/Q inequalities the true contribution of the distribution component is probably more than one half The age regressions of $C_{(A-a\ O}$ and $C_{(A-a\ O_2}-C_{(A-a\ O\ ox}$ can only partly be explained by rising a v O_2 difference (Strandell 1964) They must signify an increasing maldistribution of V_A/Q ratio with increasing age which is also in accordance with the corresponding age regression of V_D/V_T

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Cellular Localization of Monoamine Oxidase in Rat Salivary Glands

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Abstract

ALMGREN, O, N-E ANDÉN, J JONASON, K-A NORBERG and L OLSON *Cellular localization of monoamine oxidase in rat salivary glands* Acta physiol scand 1966 67 21—26

After ligation of the excretory ducts and subsequent atrophy of the parenchymal cells of the rat submaxillary and sublingual glands a severe reduction of the MAO activity was demonstrated both biochemically (more than 60 per cent decrease expressed as activity per gland) and histochemically Chronic postganglionic sympathectomy produced a much smaller diminution of the MAO activity in these glands It is concluded that the major part of the MAO in rat salivary glands occurs in the parenchymal cells

There is now general agreement that the enzyme monoamine oxidase (MAO) is of great importance in the metabolism of the naturally occurring monoamines Pharmacological, biochemical and histochemical data support the view that the MAO occurs in the sympathetic nerve terminals but the evidence is indirect (Carlsson *et al* 1957, Kopin 1964, Norberg 1965, Malmfors 1965) In the present study the cellular localization of this enzyme in rat salivary glands has been investigated by determining the activity of MAO both biochemically and histochemically after selective degeneration of either the gland cells or the sympathetic nerve terminals

Material and Methods

Adult rats of both sexes were used Atrophy of the submaxillary and sublingual glands was produced by ligation of the excretory ducts near the hilum The submaxillary and sublingual glands were post ganglionically sympathectomized by excision of the superior cervical ganglion The glands were pre ganglionically sympathectomized by the removal of 1 cm of the cervical sympathetic trunk The operations were performed under ether or pentobarbital sodium anesthesia In all cases the operation was unilateral, the intact side serving as a control

For the biochemical determinations the animals were killed about 14 days after the operations by a blow [illegible]
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TABLE I. MAO activity and weight of intact and atrophied salivary glands about 14 days after unilateral duct ligation. The enzyme activity was estimated by (1) oxygen consumption using tyramine as substrate (µmol/gland · hr), (2) dihydroxyphenylacetic acid formation from dopamine (µg gland hr), (3) [14]C-p-hydroxyphenylacetic acid formation from tyramine (µg gland hr)

Method	Atrophied gland: Weight in mg	Atrophied gland: MAO activity per gland	Intact gland: Weight in mg	Intact gland: MAO activity per gland	Atrophied/Intact gland in %: Weight	Atrophied/Intact gland in %: MAO activity
1	111	0 24	277	2 3	31	10
	67	0 95	237	2 5	28	39
	68	0 33	174	1 6	39	21
	111	0 60	—	2 3	—	26
Mean	89	0 53	229	2 2	33	24
2	120	1 0	290	37	41	3
	77	2 9	250	26	31	11
3	70	8 4	268	22	26	38
	75	5 0	258	23	29	22
	176	5 8	270	24	65	24
	81	10	276	30	29	33
	77	11	259	51	30	22
	82	15	272	42	30	36
	148	23	301	62	49	38
	113	14	305	49	37	29
	117	18	317	56	37	31
Mean	104	12	281	40	37	30

buffer pH 8.0 and the whole homogenate was incubated together with tyramine in a Warburg apparatus as described by Creasey (1956. In the second method one gland was homogenized in 0.5 ml 1/15 M phosphate buffer pH 7.5 and the whole homogenate was incubated at 37° C and oxygen atmosphere for 30 min together with 250 µg dopamine and buffer up to 3.0 ml. The reaction was stopped by the addition of 1.0 ml 10% metaphosphoric acid. The formed dihydroxyphenylacetic acid was determined spectrophotofluorometrically after organic solvent extraction and ethylene

Fig. 1. Rat submaxillary gland stained for MAO activity, using tryptamine as substrate and tetranitro-blue tetrazolium as electron acceptor.
a) No pretreatment. Rather strong specific staining for MAO activity is seen in the cytoplasm of the acini and duct cells. Mast cells in the connective tissue are recognized by their dark cytoplasm (unspecific staining). 200
b) No pretreatment. Strong specific staining for MAO activity is seen in the basal part of the duct [illegible]
MAO activity demonstrable in the parenchyma. Dark granules and mast cells without any speci[illegible] [illegible] 200
[illegible] in the well [illegible] nbranes of [illegible] nchyma in
c) and d) is seen not because of specific staining for MAO activity but by the optical characteristics of the unstained sections. 200



TABLE II MAO activity and weight of intact and postganglionically sympathectomized salivary glands about 14 days after unilateral denervation. The enzyme activity was estimated by (1) oxygen consumption using tyramine as substrate (μmol/gland · hr) (2) dihydroxyphenylacetic acid formation from dopamine (μg/gland · hr) and (3) ^{14}C-p-hydroxyphenylacetic acid formation from tyramine (μg/gland · hr)

	Denervated gland		Intact gland		Denervated/Intact gland in %	
Method	Weight in mg	MAO activity per gland	Weight in mg	MAO activity per gland	Weight	MAO activity
1	272	2 2	237	2 8	115	80
	194	3 1	243	3 9	80	80
2	285	32	277	31	103	103
	252	36	318	27	79	131
	275	22	287	22	96	98
Mean	271	30	294	27	93	111
3	246	20	292	31	84	64
	246	43	273	50	90	87
	272	34	282	40	96	85
	290	38	296	52	98	73
	280	29	291	43	96	67
Mean	267	33	287	43	93	75

diamine condensation (Andén, Roos and Werdinius 1963). In the third method one gland was homogenized in 1.0 ml chilled isotonic KCl. For the incubation 100 μl of the homogenate was mixed with 15 μl of ^{14}C-tyramine (76.4 μg/ml, specific activity 73.2 μC/mmole, 2 ^{14}C) and 250 μl 0.5 M phosphate buffer, pH 7.4, during 20 min at 37° C. The reaction was stopped by the addition of 0.2 ml 2 N HCl. The formed ^{14}C-p-hydroxyphenylacetic acid was transferred into 6 ml toluene by shaking. The radioactivity in an aliquot of the organic phase was determined in a liquid scintillation detector. Parallel experiments with either boiled enzyme or addition of a MAO inhibitor (tranylcypromine 10^{-3} M) were always performed. For control some animals were pretreated 5 hrs before sacrifice with the MAO inhibitor nialamide (500 mg/kg i.p.).

For the histochemical determinations the animals were killed by bleeding out under light ether anesthesia 7 to 14 days after the operations. Histochemical staining for MAO was performed using tetranitro-blue tetrazolium as an electron acceptor according to Glenner *et al.* (1957). For control some animals were pretreated 4 hrs before sacrifice with the MAO inhibitor nialamide (500 mg/kg i.p.).

Results and Discussion

As found in previous investigations ligation of the excretory ducts of the submaxillary and sublingual glands caused a marked reduction of the gland weights (Andén, Norberg and Olson 1965, see Table I). This atrophy is not accompanied by a corresponding loss of noradrenaline, as found both biochemically and histochemically, and therefore it is likely that the sympathetic nerve terminals are essentially intact (Andén *et al.* 1965). The biochemically determined MAO activity in these atrophied salivary glands is presented in Table I and is compared to that of the glands on the unoperated side. All the three methods used showed a considerable loss of the amount of MAO activity per gland after excretory duct ligation. In

TABLE III MAO activity and weight of intact and preganglionically sympathectomized rat salivary glands about 14 days after unilateral decentralization The enzyme activity was estimated by ^{14}C-p-hydroxyphenylacetic acid formation from tyramine (μg/gland hr)

	Decentralized gland		Intact gland		Decentralized/Intact gland in %	
	Weight in mg	MAO activity per gland	Weight in mg	MAO activity per gland	Weight	MAO activity
	284	60	281	57	101	105
	229	55	230	63	99	87
	229	60	246	63	93	95
	243	57	226	60	108	95
Mean	246	58	246	61	100	96

control experiments almost complete recoveries of MAO activity were obtained when a homogenate of a normal gland was added to that of an atrophied one This finding shows that the observed reduction of MAO activity per gland after atrophy was not due to the presence of an inhibitory substance There was, however, never a complete loss of MAO activity in the atrophied glands Pretreatment of the animals with nialamide, addition of tranylcypromine to the homogenate or boiling of the homogenate caused disappearance of this rest activity indicating that also this is due to the presence of MAO Nor could any MAO activity be demonstrated in the intact glands after these procedures

Histochemically an intense MAO activity was found in the acinar cells of the normal submaxillary and sublingual glands The staining was fairly evenly distributed throughout the cytoplasm All duct cells exhibited an intense staining in the basal parts, corresponding to the mitochondrial layer (Scott and Pease 1959), while the nuclei and the apical cytoplasm showed no staining for MAO activity (Fig 1 a and b). After ligation and subsequent atrophy of the acinar cells, practically no staining for MAO activity could be observed in the parenchyma of the glands (Fig 1 c) Pretreatment of the animals with the MAO inhibitor nialamide prevented the appearance of specific staining (Fig 1 d)

The biochemically estimated MAO activity in the salivary glands about 14 days after postganglionic sympathectomy is presented in Table II With two of the three methods used a slight reduction of the enzyme activity in the denervated glands compared to that in the intact ones was observed Histochemically no difference could be observed between the MAO activity of the denervated and the intact salivary glands It is to be noted, however, that no histochemical MAO activity was seen in sympathetic nerve terminals even in the intact glands This finding may possibly be due to the fact that the sympathetic nerve terminals are very thin and that the resolution and/or sensitivity of the present method does not allow their detection in the light microscope

Preganglionic sympathetic denervation apparently did not reduce the biochemically detectable MAO activity in rat submaxillary and sublingual glands (Table III)

It is noteworthy that in the limited material presented here there seemed to be certain differences in the results obtained with dopamine and tyramine as the substrate In the former case the reduction of the MAO activity per gland after parenchymal degeneration appeared larger whereas any loss of the enzyme activity after postganglionic sympathectomy hardly could be observed Studies on the significance of this apparent difference between dopamine and tyramine are in progress

Recently, Snyder, Fischer and Axelrod (1965) have observed a slight reduction of the MAO activity in rat salivary glands after excision of the superior cervical ganglion in agreement with our results when tyramine was utilized as the substrate Histochemically it has been demonstrated that in the superior cervical ganglion MAO activity as well as noradrenaline occur in the cell bodies of the adrenergic neurons innervating e g the salivary glands (Koelle and Valk Jr 1954, Norberg and Hamberger 1964, Norberg, unpublished data) It is thus reasonable to assume that also the terminals of these neurons in the salivary glands contain MAO The major part of the MAO activity of the salivary glands is, however, localized in the parenchymal cells as shown by both biochemical and histochemical techniques in the present investigation Studies on the functional significance of the MAO in these effector cells are in progress

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From the Department of Physiological Chemistry, University of Lund, Sweden

Intestinal Dipeptidases.

IV. Studies on the Release and Subcellular Distribution of Intestinal Dipeptidases of the Mucosa Cells of the Pig

By

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Abstract

JOSEFSSON, L and H SJOSTROM *Intestinal dipeptidases IV Studies on the release and subcellular distribution of intestinal dipeptidases of the mucosa cells of the pig* Acta physiol scand 1966 67 27—33

Dipeptidases are released very quickly (10—20 sec) from the mucosa cells of the pig small intestine

located in the supernatant fraction No conclusion can be made about the intracellular location of the enzymes because of their rapid release into the solvent The physiological interpretation of these findings is discussed

In preceding reports of this series data have been presented about the properties of various dipeptidases of the mucosa cells of the small intestine and their distribution along the gastrointestinal tract (Josefsson and Lindberg 1965 a, b, Lindberg and Josefsson 1966) Little is known about the location of their physiological action Because of results obtained in studies on other terminal digestive enzymes (Borgstrom *et al* 1957, Miller and Crane 1961 a, Dahlquist and Brun 1962, Doell and Kretschmer 1963) the concept has been widely accepted during the last years that the dipeptidases effect their physiological action within the mucosal cells of the small intestine However, results reported recently from investigations performed on the intestinal digestion and absorption of dipeptides have been in favour both for an extracellular (Dawson and Holdsworth 1962 and for an intracellular (Newey and Smyth 1962) hydrolysis Elucidation of the question would be greatly facilitated if more direct

information about the physiological properties of the dipeptidases were available

We have therefore investigated the release of the dipeptidases from the mucosa cells of the small intestine of the pig under various conditions The results are described in the present report which also includes attempts to determine the subcellular location of the enzymes by isolation of various subcellular fractions

Material and Methods

Source of the intestinal mucosa Small intestine of adult pigs were cut out immediately after slaughter and stored at 0 Less than 1 hr after the slaughter pieces (2–5 cm) taken about 100 cm from the pylorus were cut out and used for the experiments *When the mucosa was needed the pieces were cut longitudinally* and the exposed mucosa was scraped off with a glass slide

Dipeptides L-Alanyl-L-glutamic acid L-alanyl-L-proline glycyl-L-leucine and glycyl-L-valine previously characterized (Josefsson and Lindberg 1965 a Lindberg and Josefsson 1966) were used in the same concentrations as reported earlier (Josefsson and Lindberg 1965 a Lindberg and Josefsson 1966

Amino acids The amino acids were all products of Mann Research Lab New York and used in the same concentrations and combinations as described previously (Josefsson and Lindberg 1965 a Lindberg and Josefsson 1966)

Other chemicals used were of analytical grade

Assays The dipeptidase activities were determined by the spectrophotometric method in the same manner as previously described (Josefsson and Lindberg 1965 a Buffers and pH of digest mixtures were chosen for optimal conditions in the assays Josefsson and Lindberg 1965 a, Lindberg and Josefsson 1966 Unit of dipeptidase activity was determined according to previous definition (Josefsson and Lindberg 1965 b

Isolation of subcellular fractions from intestinal mucosa All procedures were made at 4 if not otherwise [illegible]

[illegible]

sediment resuspended in 0.005 M EDTA The separate fractions were assayed for dipeptidase activity and for their protein content Phase microscopy was performed on the homogenate and the different fractions and electron micrographs were taken *of the particles obtained in the sediment*

Differential centrifugation of the mucosa homogenate was made as described by Doell and Kretchmer 1962 Mucosa was homogenized with 10 vol of 0.25 M sucrose for 2 min (MSE-homogenizer 4 500 rpm The homogenate was centrifuged at 2 × 10^3 *g* min to sediment the nuclei International model HR 1 The sediment was resuspended in 0.25 M sucrose and the centrifugation was repeated The sediment was taken as the nuclear fraction The supernatants were combined and centrifuged at 1 × 10 *g* min International model HR 1 to sediment the mitochondria The sediment was resuspended in 0.25 M sucrose and the centrifugation was repeated The sediment was taken as the mitochondrial fraction The supernatant was combined and centrifuged at 6.3 × 10^6 *g* min Spinco model L to sediment the microsomal fraction and give the final supernatant The different fractions were assayed for dipeptidase activity and protein content

Procedures for releasing the activities All procedures were made at 4 Four consecutive pieces of the same size were taken from an intestine One of the pieces was placed intact in a vessel From the other three pieces the mucosa was scraped off in the usual manner and placed in separate vessels 10 vol 0.25 M sucrose were added into the vessel containing the intact piece of intestine and into two of those containing the mucosa scrapings One of the latter was stirred slowly by the aid of a magnet while the two other samples were left undisturbed At various time intervals samples were withdrawn from the different solutions and assayed for dipeptidase activity and protein content The third mucosa scraping was homogenized for 2 min MSE homogenizer 14 500 rpm with 10 vol of 0.25 M sucrose and centrifuged for 30 min (International model HR 1 15 000 rpm The supernatant was assayed for its activity and protein content

In some of the experiments performed with the intact pieces of the intestine the condition of the [illegible]

[illegible]

protein content

Protein was determined according to the procedure of Lowry *et al* 1951 using crystallized bovine ribonuclease (Armour Lot No DCO 850) for preparation of the standard curve

TABLE I Distribution of glycyl L-leucine dipeptidase activity in different fractions of brush border preparation

Fraction	Units of activity	Recovery %
Filtrate	252	100
Sediment	19	7 5
Supernatant	228	90 5

Results

Assays of subcellular fractions of mucosa cells

Analyses performed on the different fractions obtained during the preparation of the brush border revealed that nearly all the dipeptidase activity was present in the supernatant fraction The brush border fraction retained less than 10 % of the activity as shown in Table I The results do not necessarely eliminate the brush border as a tentative location for the dipeptidases, but merely indicate that the enzymes are released very rapidly from the cell debris Considering the nature of the sediment obtained in the preparations it is questionable if it really consisted mostly of brush borders Although phase microscope examination showed a picture similar to that presented by Miller and Crane (1961 b), the electron micrographs of the particles could not verify the presence of large numbers of brush border structures in the sediments Since it is unlikely that the fractionation procedure gives pure brush border fractions it may be difficult to obtain them in an electron micrograph

TABLE II Dipeptidase activities in subcellular fractions of pig intestinal mucosa homogenate

		Activity								
		L-Alanyl-L-glutamic acid			Glycyl L-leucine			Glycyl L-valine		
Fraction	Protein mg	Units	Units/mg protein	Recovery %	Units	Units/mg protein	Recovery %	Units	Units/mg protein	Recovery %
Whole homogenate	151	420	2 8	100	1 812	12 0	100	592	3 9	100
Nuclear	78	27	0 4	6 4	85	1 1	4 7	–	–	–
Mitochondrial	14	10	0 7	2 4	33	2 4	1 8	5	0 4	0 8
Microsomal	15	12	0 8	2 9	47	3 1	2 6	7	0.5	1 2
Soluble	43	374	8 7	89 0	1 631	37 9	90 0	558	13 0	94 5
Total				100 7			99 1			96 5

TABLE III Glycyl-L-leucine dipeptidase activity recovered in the supernatant when homogenizing pig intestinal mucosa with various solvents

Solvent	Activity	
	Units	Units/mg protein
Water	925	12.1
0.1 M NaCl	1,305	13.4
0.25 M sucrose	1,520	14.4
0.005 M EDTA	365	5.7

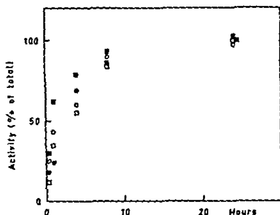
The rapid liberation of the dipeptidases from mucosal cell debris was confirmed by the results found when the various fractions from the differential centrifugation were analyzed. The results are presented in Table II. Most of the activities were obtained in the supernatant and only traces of the various activities were retained in the other fractions. These findings also confirmed the results reported recently by Robinson (1963) in his study of the subcellular fractions of rat intestinal mucosa homogenate, where the dipeptidases were assayed with the ninhydrin method. They are also in agreement with previous observations on homogenate of other tissues (Rademaker and Soons 1957, Hanson and Blech 1959). Variations of the homogenizing procedure both in respect to time and force gave no difference in the distribution of the activities.

Studies on the release of the dipeptidases

The influence of various solvents used in the homogenizing procedure was studied in respect to the amount of activity obtained. The results of these studies, preliminary to an attempt at isolating the enzymes in pure form, are shown in Table III. 0.25 M sucrose was found to be the most effective of the solvents studied. The small amount of activity obtained with EDTA is due to its capacity of binding bivalent metal ions, known to influence the dipeptidase activity greatly.

Investigations of variation in the homogenizing procedure, involving different homogenizers (MSL and Potter Elvehjem type), various volumes of solvent and different time and force, showed those factors to be of no influence on the yields of dipeptidase activity from the mucosa scrapings.

The easy liberation of the dipeptidases from the mucosa homogenate incited the investigations performed on the release of the enzymes from the cells into the surrounding solvent. It was then observed that most of the activities were liberated very rapidly into the solvent when mucosa scrapings were used. When slow magnet stirring was included into the experiment more than 50 % of the total activity was released within a period of 10—20 sec and the release was complete after 40 min. The rate of liberation decreased when no stirring was performed but the same amount of the various activities was obtained in both cases. When intact pieces of the intes-



●
○ L-Alanyl L-proline
■ Glycyl L-leucine
□ Glycyl L-valine

tine were investigated, the rate of liberation of the dipeptidases was very similar to the rate observed with the mucosa scrapings and no difference was found in the yield of the activities Results from the typical experiment are shown in Fig 1 The histological examinations revealed that the membranes of the mucosa cells were intact during the whole time of the experiments

Discussion

If the rapid liberation of the dipeptidases is considered first, it is evident that they differ markedly in this respect from the observations previously made on other intestinal hydrolases Thus the intestinal disaccharidases are so firmly attached intra cellularly that special procedures, involving proteolytic digestion must be used to perform their solubilization (Borgstrom and Dahlquist 1958 Auricchio *et al* 1963) The observations that the acid hydrolases are located in the lysosomal fraction (Novikoff 1961) and that the alkaline phosphatase is associated to the brush border (Moog 1962) also indicate a more stable intracellular association of these enzymes

What this looser nature of the dipeptidases means in physiological terms is more difficult to interpret The present results are in support of an extracellular location of the dipeptide hydrolysis and thus in agreement with the observations made by Dawson and Holdsworth (1962) that no dipeptides enter the epithelium cells of the small intestine under physiological conditions When the results are related to the circulation experiments reported by Newey and Smyth 1962 which led them to the opposite conclusion it is obvious that more careful quantitative determination of the dipeptidase activity must be included in their experiments before they can be usable for conclusions about the mechanism of the dipeptide hydrolysis The rapid release of the dipeptidases from the mucosa cells also calls attention to the possi-

TABLE IV Glycyl L-leucine dipeptidase activity extracted from pig small intestine with 0 25 M sucrose solution under various conditions at 4°

Condition	Activity	
	Units	Units/mg protein
Mucosa, homogenized 2 min at 14 500 rpm	3,525	13 8
Mucosa, extracted 1 h with stirring	3 180	57.1
Mucosa extracted 24 h without stirring	3,210	78.5
Whole intestine, extracted 24 h without stirring	3 030	63 8

bility that the enzymes are secreted into the intestinal lumen under physiological conditions Such a consideration has a special interest since it recently has been demonstrated that the "undifferentiated" cells of the crypts of Lieberkühn of the small intestine have secretory activity (Trier 1964) Further investigations of the question will be necessary before any certain conclusion can be made.

The results obtained in the analysis of the isolated subcellular fractions of the mucosa cells are also easily explained by the rapid liberation of the enzymes It is therefore evident that differential centrifugation of aqueous homogenate will not be of any help in elucidating the intracellular location of the dipeptidases To obtain information about this question it will be necessary to use experimental conditions where the cells or the cell constituents have no contact with polar solvents

When regarding the different procedures used for the liberation of the activity in view of preliminary purification steps, it was of interest to compare not only the total yield of activity obtained, but also to relate the amount of activity obtained to the total amount of protein extracted under the various conditions Such figures, obtained from experiments performed on consecutive intestinal pieces of the same size, are arranged in Table IV It is evident that homogenization released most activity but gave rather low figures in regard to specific activity, while the other procedures, though resulting in somewhat lower yields, showed rather high specific activity, thus making them more suitable to use in this respect The time convenience of the procedure, involving slow stirring of a mucosa scraping, may promote this procedure because of the unstable nature of the enzymes

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From the Department of Pharmacology, University of Lund, Sweden

Influence of Ionic Environment on Acetylcholine Release from the Motor Nerve Terminals

By

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Abstract

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Acetylcholine release from motor nerve terminals in rat diaphragm was estimated as the frequency of miniature end plate potentials (m.e.p.p.s). K^+ 20 mM induced sustained rapid release of m.e.p.p.s which was slow in onset. With higher K^+ concentration m.e.p.p. frequency rose rapidly. Very high release rates induced by 40 and 50 mM K^+ were not maintained but declined to a lower sustained level. Augmented m.e.p.p. release could be inhibited by increasing the Ca^{++} concentration above normal. Reduction was approximately 80 % with 16 mM Ca^{++}. Reduction of Na^+ to 1/3 doubled the m.e.p.p. rate. Increase in Ca^{++} raised the rate further in 5 mM K^+ but reduced it in 20 mM K^+. The mechanism of low sodium augmentation of release is obscure but may involve inverse interaction with calcium at an early step in ACh release. Elevation of Ba^{++} to 16 mM gave a transient decline in m.e.p.p. frequency augmented to high level with 20 mM K^+, presumably by displacing residual bound calcium. It did not reduce the frequency when Ba^{++} had replaced Ca^{++} in the bathing solution for longer periods. The effects of calcium are discussed and the similarities between excitation transmitter release and excitation-contraction coupling are pointed out.

It is well established that electrical or potassium induced depolarization of the motor nerve terminals causes a great increase in the rate of release of acetylcholine (ACh) packages recorded as miniature end-plate potentials (m.e.p.p.s.) (del Castillo and Katz 1954, Liley 1956 b, Katz 1962). Liley (1956 b) and Hubbard (1961) have shown that calcium is necessary for this augmented release to occur and that excess magnesium inhibits it. Kelly (1965) and Birks and Cohen (1965) observed that an action potential in the motor nerve released more ACh packages when the sodium concentration was reduced. Hubbard (1961) showed that quadrupling the calcium concentration about doubles the resting frequency of m.e.p.p.s.

During the investigation of the effect of ouabain on the transmitter release from the motor nerve terminals this drug was found to increase m.e.p.p. frequency in the presence of normal calcium concentration (Elmqvist and Feldman 1965 b). Thus

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augmented release was not inhibited by magnesium but high calcium concentrations reversibly reduced the augmented frequency by about 90 %

These findings prompted a further investigation of ionic influences on transmitter release at the motor nerve terminals.

Methods

Intracellular recordings from the end plate region of single muscle fibres from the left hemidiaphragm of rats (100—200 g) were undertaken as previously described (Elmqvist and Feldman 1965 a, b) All solutions contained choline 5×10^{-4} g/ml (3.5×10^{-6}M) and experiments were carried out at 32° C

increase When K^+ was increased to 20 mM no change in other components of the bath was made This modified solution was approximately 10 % greater than "normal" in osmolarity A preliminary study of the effect of this increase in tonicity upon the m e p p rate was therefore undertaken In the

1965 a)

The rate of ACh release was estimated by the frequency of m e p p.s at the muscle end plate Since the amplitude of the potentials declines when the fibre is depolarized (Katz and Thesleff 1957), when the external sodium is reduced (Fatt and Katz 1952, Takeuchi and Takeuchi 1960) or when the calcium concentration is elevated (Takeuchi 1963) only records with very low base-line noise in which the m e p p s had a rise-time of 1 5 msec or less were analyzed Counts were made from the ink-written record At lower rates a minimum of 100 potentials was counted for each determination of the rate, at higher frequencies the potentials appearing in one second were counted

Results

Potassium

On the basis of experiments with raised potassium concentrations Liley (1956 b) arrived at the conclusion that the release of transmitter by the nerve impulse was quantitatively determined by the depolarization of the motor nerve terminals

Recent studies (Elmqvist and Quastel 1965 a, Gage and Quastel 1965 a) have suggested that the increase in m e p p frequency is not rapid enough to support this hypothesis In human intercostal muscle higher potassium concentrations gave more rapidly achieved high release rates which however were not sustained (Elmqvist 1965) Similar experiments with potassium concentrations from 5—50 mM were

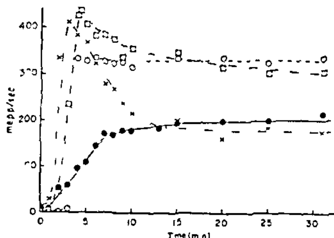


Fig 1 M e p p frequency at single rat phrenic neuromuscular junctions after bathing solution was changed at time zero from one containing 5 mM K to
20 mM K (●——),
30 mM K (○— — —)
40 mM K⁺ (□— —) and
50 mM K (× — — —)
All solutions contained 2 mM Ca²

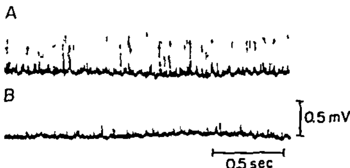


Fig 2 Intracellularly recorded m e p p s at a rat phrenic neuromuscular junction in 20 mM K , A in 2 mM Ca² (RP55 mV) B 10 min after increasing Ca² to 16 mM (RP51 mV) Calibration 0.5 mV 0.5 sec

undertaken in the rat diaphragm With the preparation in normal bathing solution (5 mM K) a single fibre was impaled at the end plate and the resting frequency observed for at least 5 min The solution was then changed to one containing the raised potassium ion concentration and the m e p p s were continuously recorded for at least 30 min (Fig 1) Although the time required to reach a steady frequency in 20 mM K was 15—20 min, with higher concentration peak frequencies were achieved within the first 5 min The peak frequencies obtained with 40 and 50 mM K^+ were not maintained, but declined and had achieved a somewhat lower constant level after about 10 min

Calcium

The effect of raised calcium ion concentrations on augmented release was studied in solutions containing 20 mM K This potassium concentration was chosen as it gave a high release rate without excessive reduction in m e p p amplitudes (Katz and Thesleff 1957) The preparations were equilibrated for 30 min in the bathing solution containing 20 mM K^+ and 2 mM Ca^{2} After obtaining m e p p frequency at 10 end plates the microelectrode was left in a fibre and the solution changed to one containing the desired raised calcium level After the time course of the effects of this solution change was observed for 30 min the miniature frequency was deter-

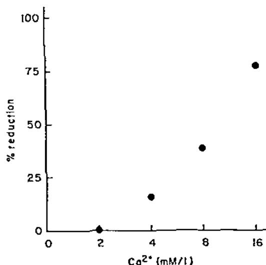


Fig 3 Reduction in augmented m e p p frequency obtained by various Ca^{2+} concentrations in the presence of 20 mM K^+ % reduction was calculated from the relationship % $= (f_2 - f_n/f_2) \times 100$ where f_2 is frequency in 2 mM Ca^{2+} and f_n in 4 8 and 16 mM respectively Note logarithmic scale in abscissa

mined in another 10 fibres The microelectrode was again left in a fibre and the bathing solution was changed back to the original one This process was repeated at least twice in the same preparation The m e p p rates observed at different periods in the same bathing solution did not differ significantly and the results were therefore pooled

There was a reduction in the augmented frequency when the calcium concentration was raised from 2 mM to 4, 8, or 16 mM (Fig 2) In each specimen the reduction in m e p p frequency was determined as the percentage of the frequency in 2 mM Ca^{2+} As is shown in Fig 3 there was an approximately linear relationship between the percentage reduction and the logarithm of the calcium ion concentration The effect of raising calcium ion concentration in reducing frequency, or restoring Ca^{2+} to normal (2 mM) in increasing the rate, was complete within 3—5 min Elevated calcium ion concentration was associated with a decrease in the amplitude of the miniature end plate potentials but a quantitative study of this phenomenon was not undertaken

Barium

It has recently been shown that barium can restore and maintain the release of ACh packages completely abolished by prolonged calcium deprivation (Elmqvist and Feldman 1965 a) The ability of barium to substitute for calcium in the inhibition of release was therefore studied Specimens were dissected and mounted in normal bathing solution and then placed in a bathing solution containing 20 mM K^+ and 2 mM Ba^{2+} and experiments identical to those with raised calcium ion concentration were performed using 2 and 16 mM Ba^{2+} When the barium concentration was increased to 16 mM for the first time there was a prompt decline in rate to about 1/3 within 3 min During the next 7 min the rate returned to about 80 % of the frequency in 2 mM Ba^{2+} and remained at that level After 30 min in 16 mM Ba^{2+}

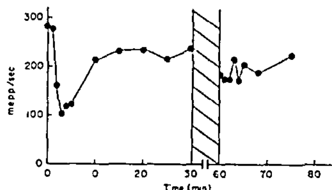


Fig 4 M e p p frequency at single muscle end plate in 20 mM K After 1/2 hour in a solution containing 2 mM Ba²⁺ (no Ca²⁺) The Ba²⁺ concentration was changed to 16 mM at time zero At 30 min Ba²⁺ was reduced to 2 mM (cross-hatched area) At 60 min Ba²⁺ was again increased to 16 mM

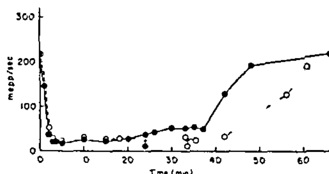


Fig 5 Different effects of Ba²⁺ and Ca²⁺ 16 mM on m e p p. frequency in 20 mM K followed at a single neuromuscular junction After 2 hours exposure to Ba²⁺ 2 mM and 30 min in Ba²⁺ 16 mM Ca_2 16 mM was substituted at time zero At the arrows Ba²⁺ 16 mM replaced the Ca²⁺ The records are continuous (●—— first hour O — — — second hour)

a reduction to 2 mM Ba²⁺ caused no change in m e p p frequency After an additional 30 min an increase in Ba²⁺ concentration to 16 mM did not alter the frequency (Fig 4) The frequencies observed after 30 min in 2 mM Ba²⁺ were 250 ± 9 5 m e p p s/sec (mean ± S E 21 fibres) and in 16 mM Ba²⁺ 236 ± 5 8 (13 fibres)

When a preparation had been exposed to 16 mM Ba²⁺ and 20 mM K for 2 hrs the frequency was high Replacement of the barium by calcium (16 mM) caused a prompt and profound decrease in the rate which was maintained for at least 30 min Returning to 16 mM Ba²⁺ brought about a restoration of the rate beginning in about 10 min and requiring 20 min for its full effect These effects could be observed repeatedly on serial observations in a single fibre (Fig 5)

Sodium

Birks and Cohen (1965) and Kelly (1965) have shown that the quantum content of the end plate potentials was increased when the ambient sodium concentration was reduced They suggested that this phenomenon was effected by a sodium calcium antagonism As there are many similarities between impulse elicited release and spontaneous release (Katz 1962) the effects of lowered sodium concentration on spontaneous release were studied The effects of elevated sodium concentration were not evaluated as the hyperosmolarity necessarily obtained in such solutions greatly increased m e p p frequency (Liley 1956 a) When Na concentration was reduced

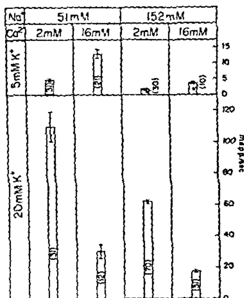


Fig 6 M e p p frequencies in various ionic environments. Bar height indicates frequency, vertical line is ± SEM Number in brackets gives number of fibres in which rate was counted

to 51 mM (1/3 normal) the frequency of the m e p p s was increased by a factor of 3 7

The rate returned to the normal level when the sodium concentration was restored When the calcium ion concentration was increased to 16 mM in the presence of low sodium a further increase by an additional factor of 2 7 occurred as opposed to the decrease in frequency seen when m e p p frequency was augmented by potassium (Fig 6) When the sodium concentration was reduced to one-third in the presence of 20 mM K^+ the m e p p frequency was about doubled This effect occurred promptly and replacement of the sodium led to a rapid reduction A low sodium environment neither enhanced nor inhibited the ability of elevated calcium concentrations to reduce the m e p p frequency when increased to a high level by 20 mM K^+ Specimens exposed to a low sodium, 2 mM Ca^{2+}, and 20 mM K^+ environment showed a prompt reduction in frequency when the Ca^{2+} was increased to 16 mM The rate declined to 24 % of the previous value within 4 min and remained at this level for at least 1 hr, in contrast to the rise in frequency caused by Ca^{2+} increase in the presence of low sodium and normal K

Chloride

The possibility that the lower frequencies observed in normal sodium and high calcium were caused by differences in chloride concentrations was evaluated When two-thirds of the chloride was replaced by nitrate in otherwise normal bathing solution no change in m e p p frequences was observed. The frequency was augmented by 20 mM K^+ and this augmented release was inhibited by increasing Ca^{2+} to 16 mM utilizing $Ca(NO_3)_2$ The frequencies observed under these conditions did not differ from those observed when the solutions were prepared from only chloride salts

Some increase in the amplitude of the m e p p s was observed in nitrate solutions Some experiments were performed in chloride free solutions using commercial sodium and potassium ethanesulfonate salts The resting rates in normal Ca^{+}, K, and Na^{+} concentrations were about 10—12 sec (5 times normal) The effects of changing potassium sodium and calcium concentrations were qualitatively similar to those observed in the presence of chloride ions

Discussion

Effects of high potassium ion concentration

Elmqvist and Quastel s (1965 a) and Gage and Quastel s (1965 a) observations that the increase in m e p p rate produced by 20 mM K^{+} has a slow onset have been confirmed The peak frequencies on changing to higher K concentrations however appear within the wash-out period These phenomena in the rat phrenic diaphragm preparation are similar to those seen in the human intercostal muscle (Elmqvist 1965) The reason for the slower effects with the lower concentration is not clear The failure of the high release rates to be sustained may reflect depletion of preexisting pools of ACh units similar to those predicated by Elmqvist and Quastel (1965 b) Because of such depletion the m e p p rate in the very high potassium ion concentration may never be able to reach the level which it would otherwise finally attain

Sodium and Calcium

The ability of sodium to increase the m e p p frequency parallels the increase in quantum content induced by lowered sodium on the impulse triggered end plate potential Birks and Cohen 1965 Kelly 1965 Gage and Quastel (1965 b) showed an increase in m e p p frequency when sodium was decreased A reduction in sodium concentration in the present study to 1 3 caused an increase in rate of 2—3 times or an increase similar to that produced by increasing the calcium concentration to 30 mM (Elmqvist and Feldman unpublished observations) or with caffeine and added calcium Elmqvist and Feldman 1965 a In the "resting 5 mM K situation the effects of lowering Na-concentration and increasing Ca-concentration were additive but when release was augmented by 20 mM K increasing the Ca-concentration to 16 mM reduced the frequency from 110 sec to about 20 sec (Fig 6) Similar results have been obtained by Douglas and Poisner 1964 on the release of vasopressin from the posterior pituitary gland The interactions of sodium and calcium ions are in accordance with the hypotheses of Birks and Cohen 1965 and of Kelly (1965 that there is in the motor nerve terminal a competition between sodium and calcium as proposed by Niedergerke 1963 in the frog heart

It remains however to be explained how the elevated Ca^{+} concentration inhibits the release induced by depolarization Kuriyama (1964) has shown that elevation of Ca^{+} hyperpolarized guinea pig vas-deferens muscle We have not observed such a hyperpolarization in the rat diaphragm surface fibers either in normal or raised K^{+}-concentrations whether or not chloride or ethane sulfonate was the anion present However the polarization of the motor nerve terminals is not known directly An

other direct membrane effect which would limit ACh release is that the increased ability, induced by depolarization, to allow ACh to pass through the membrane is reduced because of "stabilization" of the membrane by excess Ca^{2+} (Shanes 1958 a, b) More complicated hypotheses for the role of calcium in release of ACh packages and inhibition of this release may also be postulated The release mechanism which needs calcium to be activated may, for instance, be inhibited by an excess of the ion Alternatively calcium may have opposing effects on different steps in the release process

It is of interest that there are many similar features between the factors involved in transmitter release and in the excitation contraction coupling in striated and cardiac muscle, and some of the mechanisms proposed for the excitation-contraction coupling (see Edman 1965) may also apply to the release of transmitter substances form the nerve terminals and to the release of other secretory products (Douglas and Poisner 1964)

Barium

It is clear that Ba^{2+} can substitute for Ca^{2+} in the process of ACh release (Elmqvist and Feldman 1965 a) Fig 4 demonstrates that it also may displace some form of inactive Ca^{2} which can participate in both the inhibiting and ACh releasing processes, but that it cannot induce inhibition itself, even when present in high (16 μM) concentration (Fig 4, and 5) Displacement of calcium by divalent ions, but not Ba^{2+}, has been postulated by Frank (1962) in the contraction coupling process in the frog toemuscle, and by Aidley (1965) in the locust In both these systems, however, Ba^{2+} was ineffective in either replacement or displacement of calcium Bohr (1964) reviewed the action of Ba^{2+} on smooth muscle and discussed the parallel and potentiating effects of this ion with Ca^{2+} It is unlikely that the hyperpolarization of the ganglionic neurone soma exposed to high Ba^{2+}, reported by Nishi and Soeda (1964), occurred at the motor nerve terminals under the conditions described in the present study as such an effect would give a reduction in m e p p frequency (Liley 1956 b)

As the rate of ACh release is low when Ba^{2+} has replaced Ca^{2+} and can increase when the terminal is depolarized it must be able to replace Ca^{2+} at each step in the chain of reactions leading to release of the transmitter

Chloride

No evidence was found that the effects ascribed to change in the cationic environment reflected alteration in the chloride concentration Replacement of some or all of the chloride by anions of limited permeability did not alter the observations made when cations were changed from one to another concentration

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Acta physiol. scand 1966 67. 43—49

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The Loss of Certain Cellular Components from Human Erythrocytes during Hypotonic Hemolysis in the Presence of Dextran

By

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Abstract

Hjelm, M, S G Östling and A E G Persson *The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran* Acta physiol scand 1966 67 43—49

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Marsden, Zade Oppen and Johansson (1957) reported that human erythrocytes suspended in a hypotonic solution containing dextran did not release as much hemoglobin (Hb) as cells at the same tonicity without dextran It was found that the dry masses of the cells were distributed unimodally around a new lower mean value after hemolysis (Since the stroma and components, other than Hb, constitute only about 5 per cent of the unhemolysed red cell dry mass, the latter can be used as a fair approximation of the Hb content, provided this is not too low) These observations were interpreted as indicating that partial liberation of Hb from all, or nearly all, the red cells occurred under such conditions A close relationship was found between the Hb escaping and the dextran concentration in a hypotonic solution which would cause complete hemolysis in the absence of dextran (Zade Oppen 1960 a) Thus it was possible to predict the mean loss of Hb from the erythrocyte population occurring with a particular dextran concentration in the hypotonic system

It was also found that the cells took up dextran under these conditions (Marsden and Östling 1959) Analysis of the molecular weight distribution of the dextran fixed in the red cells showed a relative overweight of low molecular dextran in the cells There appeared to be an upper limit of permeation at a molecular weight of

about 300,000 to 400,000, though this figure must be regarded as somewhat uncertain It is perhaps interesting to note that Gerhardt and Black (1961) found a similar upper exclusion limit in the walls of bacterial spores

In the present investigation the hypotonic dextran system has been utilized for studying the disappearance of molecules, other than Hb, from human erythrocytes Catalase, glucose-6-phosphate dehydrogenase, hexokinase and lactate dehydrogenase were chosen as high molecular weight substances while adenosine-triphosphate and glutathione served as low molecular weight indicators

Materials and Methods

Venous blood samples were collected in heparinized tubes from healthy blood donors at the Blood Transfusion Service (By kind courtesy of Dr C. F Hogman), University Hospital, Uppsala Phosphoglycerate kinase, phosphoglyceraldehyde dehydrogenase, ATP, NADP and NADH were obtained from Boehringer & Soehne, Mannheim, West Germany Methylglyoxal was obtained as a crude preparation from Kemikalieaktiebolaget KEBO AB Stockholm, Sweden and was purified by steam distillation. Dextran (Dextran 250® $M_w = 250\,000$ $M_n = 123\,000$) was a generous gift from AB Pharmacia Uppsala, Sweden All other chemicals were of reagent grade

General procedure

content of the hemolysate The hemolysed cells were washed in the isotonic buffer solution and finally suspended in a small volume This cell suspension was used for the analyses described below

Partial hemoglobin liberation from erythrocytes

All procedures were done at + 4 °C The heparinized whole blood sample was centrifuged at 100 × g for 5 min (Nikkila 1962 The plasma and leucocytes were removed The erythrocytes were suspended in an isotonic (289 mOsm see Hendry 1961) buffer solution pH 7.4 of the following composition NaCl 116 mM KCl 4 mM $MgCl_2$ 5.5 mM NaH_2PO_4 and Na_2HPO_4 20 mM (= 43 n Osm) (Hendry 1961 and glucose 10 mM The osmolarity of the solutions was checked by measuring the freezing point depression with an osmometer Advanced Instruments model 64-31) After centrifugation at 1500 g for 10 min the supernatant was discarded and one part of the sedmented erythrocytes (hematocrit about 90 per cent was mixed with an equal part of the washing solution One volume of the resulting suspension equilibrated with atmospheric air was rapidly mixed with 19 volumes of a hypotonic solution of the following composition

4—10 per cent (w v) dextran (measured polarimetrically) in a hypotonic (65 mOsm without dextran) buffered saline pH 7.4 consisting of NaCl 5 mM KCl 5 mM NaH_2PO_4 and Na_2HPO_4 20 mM This constituted the hemolysing system

The total Hb concentration in this system (partially hemolysed red cells + liberated Hb was determined as methemoglobincyanide by the method of Zade-Oppen (1960 b) after complete hemolysis of a small sample in destilled water diluted 1 41

The following abbreviations are used ATP adenosine triphosphate NADH reduced diphosphopyridine nucleotide, NADP, oxidized triphosphopyridine nucleotide, GSH reduced glutathione, GSSG, oxidized glutahione, Cat, Catalase, G 6-PDH glucose-6-phosphate dehydrogenase, HK, hexokinase, LDH, lactate dehydrogenase, Hb, hemoglobin

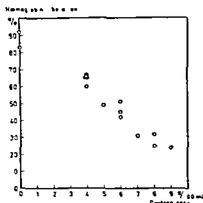


Fig 1 Relation between hemoglobin liberation and dextran concentration in the hypotonic buffer solution

supernatant after recentrifugation of the supernatant at *104 000 × g for 20 min* The loss of cells was small and very unlikely to have seriously affected the results since the average amount of Hb remaining in the cell fractions before the first washing and after the second was within experimental error the same

Preparation of the samples for determination of enzyme activities glutathione and adenosine triphosphate concentrations

Aliquots of the final erythrocyte suspension were stored in liquid nitrogen thawed at 0 °C and completely hemolyzed in hypotonic buffer The hemolysate was used for the determination of enzyme activities Another volume of the suspension was added to 10 volumes of ice cold 0 6 M

dorf photometer connected via an Eppendorf scale expander to a recorder (Philips model PR 2210 U) or a Beckman DB spectrophotometer with recorder (Sargent model SR)

Results

1 *The relation between dextran concentration and hemoglobin liberation*

The relationship is shown in Fig 1 There appears to be approximate linearity at least between 2 and 7 per cent dextran The straight line in this region was calculated by the method of least squares

2 *The relation between the dextran concentration and escape of different sized molecules during partial hemoglobin liberation*

In Fig 2 the loss of certain substances is shown in relation to the dextran concentration in the hypotonic solution The Hb release shown in Fig 1 is also red n

about 300,000 to 400,000, though this figure must be regarded as somewhat uncertain It is perhaps interesting to note that Gerhardt and Black (1961) found a similar upper exclusion limit in the walls of bacterial spores

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General procedure

A measured volume of washed human erythrocytes was suspended in a relatively large volume of a hypotonic buffer solution containing varying concentrations of dextran which regulated the amounts of Hb which were released in hypotonic hemolysis The tonicity was restored with a slightly hypertonic buffer solution The Hb liberation was calculated as the percentage of the Hb found in the clear supernatant after the cells had subsequently been spun down in relation to the total Hb content of the hemolysate The hemolysed cells were washed in the isotonic buffer solution and finally suspended in a small volume This cell suspension was used for the analyses described below

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The total Hb concentration in this system partially hemolysed red cells + liberated Hb) was determined as methemoglobincyanide by the method of Zade Oppen (1960 b) after complete hemolysis of a small sample in destilled water diluted 1 41

The suspension was adjusted to isotonicity after 30 to 60 min by adding 180 volumes of a hypertonic (315 mOsm) buffered saline pH 7 4 of the following composition NaCl 130 mM KCl 4



ATP ... NADH ... diphos
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TABLE I Recovery of lactate dehydrogenase in cell fraction and supernatant after partial hemoglobin liberation in one experiment

	Enzyme activity in international units				
Hemoglobin loss per cent	Activity per 10^{11} cells in final suspension	Activity in total volume of final suspension	Activity in total volume of first supernatant	Sum of activities in first supernatant and final suspension	Recovery of enzyme activity in first supernatant and final suspension combined per cent
0	236	10 5	0	10 5	100 (reference value)
0	255	10 5	0	10 5	100 (reference value)
66	126	5 4	3 3	8 7	83
66	131	5 6	1 1	6 7	64
66	136	5 8	1 8	7 6	72

The magnitude of the loss of a substance will depend on whether its movement is restricted spatially or temporally It may be fixed to some structural part of the cell which will prevent some or all of it escaping It may also be complexed to some other component with the result that the complex escapes at a different rate Independently of any restriction imposed by a barrier such as a membrane, differences in diffusion rates will mean that the smaller the molecule the faster will it escape from the cell and as the hemolytic process is of a transient nature the limited time available may be expected to result in differences in relative final concentrations in the cell Furthermore this effect will be exaggerated if the *transient increase in porosity itself* also exerts a molecular sieve effect (*e g as with a heteroporous membrane*)

If the increased porosity of the cell were of infinite duration allowing equilibration of all permeant species a cut off might be observed, indicating the maximum size of the "pore" Molecular species smaller than a critical 'pore size" would thus have no restriction on their escape from the cell while larger molecules would not be able to escape *In the present instance, however, the porosity appears to be of too* short a duration to allow equilibration and thus the pore size distribution may tend to impose its own graded sieving effect For example if there is heteroporosity there *is, other things being equal, a greater area available for the diffusion out of* small than large molecules It must be emphasized however, that the results shown here do not necessarily indicate the presence of a membrane sieving effect

Furthermore it is quite possible that bulk flow of water may occur during hemolysis An outward bulk flow would tend to give similar values for the relative losses of different substances An inward bulk flow opposing an outward flux of solutes due to diffusion, might tend to restrain preferentially the escape of larger solutes

The localization within the cell of the molecules studied is rather uncertain The ghosts *remaining after partial hemolysis in hypotonic dextran are almost certainly* more or less intact cells Teorell (1952) for example showed that erythrocyte ghosts produced by mild hyposmotic trauma can function as osmometers, and Stein (1956)

TABLE II Molecular weights of components studied

	Molecular weight	Reference
Cat	220 000 (human)	Herbert and Pinsent 1948
G-6-PDH	74 000 (guinea pig)	Andrews, 1962
Hb	64 000 (human)	Braunitzer et al 1961
HK	96 000 (yeast)	Kunitz and McDonald 1946
LDH	110—120,000 (pig)	Wieland, Duesberg and Determan, 1963
ATP	507	
GSH	307	
GSSG	613	

found that the transport of glycerol and other non-electrolytes was similar in ghosts and unhemolysed cells Many papers have also been published on the active transport of cations in ghosts showing their ability to accumulate and retain potassium (cf Passow 1964) Bartlett (1958) pointed out that the usual way of obtaining "stroma" by osmotic hemolysis is not a very good way of studying enzyme localization The interpretation of the results reported here will be much more difficult if we have to consider that some of the substances are fixed to immovable sites in the cell It is generally regarded as difficult to remove all the Hb from a cell but Dodge, Mitchell and Hanahan (1963) showed that Hb can be removed completely under special conditions If the red cell is considered as consisting of a thin bounding membrane containing an interior which is relatively structureless, the behaviour of Hb can then be regarded as representative for escaping species The mass of Hb in the cell is so large that even if the inner surface of the membrane fixed a complete layer of Hb molecules this would constitute only to about 1—2 per cent of the Hb originally present in the cell and thus does not introduce a serious source of error

In Table II values are given for molecular weights of the substances analysed These values are compiled from the literature and must in some cases be regarded as rather approximate The molecular weights values given for HK and Cat are derived from ultracentrifugation data In gel filtration through Sephadex® G-100 Cat is slightly more excluded then LDH which has a molecular weight of 110,000—120,000 (Aebi *et al* 1964) The values for LDH and G-6-PDH have been calculated from gel filtration data

Cat and LDH are larger than Hb and escape to a less extent than does Hb HK and G-6-PDH have sizes similar to Hb and show a similar escape pattern The tripeptide GSH has relatively small molecular dimensions (both in its reduced and oxidized forms) and leaves the cell to a greater extent than Hb The same is true for ATP although the decrease in concentration could also be explained by an increased ATP-ase activity This matter has not been seriously investigated but seems somewhat unlikely since there was no increase in adenosine diphosphate and adenosine-monophosphate concentrations wich were measured in one case

Taking all the components studied into account it appears that the relative amount

which escaped was inversely related to molecular size In view of this relationship it is tempting to suggest that the greater part, at least, of each of these substances is not immovably fixed within the cell Further, it appears that this method could be used for producing erythrocytes with reproducible differences in the intracellular composition Such cells might be useful in studies of metabolic regulation mechanisms, especially of the glycolytic system

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Serotonine and Temperature Control

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Abstract

ANDERSSON B M JOBIN and K OLSSON *Serotonine and temperature control* Acta physiol scand 1966 *67* 50—56

hypothalamus

Feldberg and Myers (c f Feldberg 1965) have recently studied the effects on body temperature on injections of 5 hydroxytryptamine (serotonine 5 HT) and catecholamines into the brain ventricular system of the cat They found that injections of 5 HT caused a marked and sustained rise in rectal temperature whereas catecholamines had the reverse effect On the basis of these results Feldberg and Myers have suggested that body temperature is the outcome of a fine balance in the release of catecholamines and of 5 HT in the hypothalamus

Since this new concept of temperature control in some respects seemed contradictory to observations on hormonal factors involved in temperature regulation in the goat (*cf* Andersson Gale and Hokfelt 1964) the experiments were repeated in the latter species

Material and Methods

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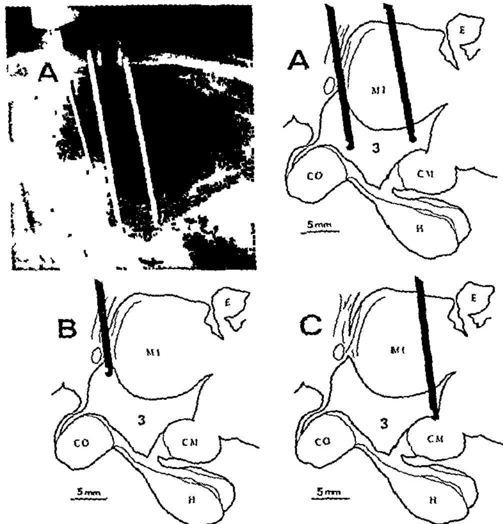


Fig. 1. *Positions of the cannulae used for injections of 5-HT and of catecholamines into the third brain ventricle of goats A, B and C.*

Upper left: Lateral X-ray picture of the skull of goat A showing the two cannulae and a thermocouple needle (T) placed laterally in the forebrain and used for recordings of brain temperature.

Upper right: A drawing of a mid-sagittal section through the diencephalon to show the position of the cannulae tips in the third brain ventricle (3) of goat A.

Below: Similar drawings to show the positions of the cannulae in goats B and C.

CO	= Chiasma opticum	H	= Hypophysis
CM	= Corpus mammillare	MI	Massa intermedia
E	Epiphysis	3	Third brain ventricle

3 days or longer in each animal. The substances used for injection: ... hydroxytryptamine creatinine sulphate, noradrenaline bitartrate an (a lcenal ne were dissolved in physiological saline immediately before the injection. The volume injected was 0.2 ml followed by a 0.2 ml saline wash (corresponding to the dead space of the injection system). The exogenous pyrogen used in some experiments was pseudomonas polysaccharide (Piromen, Travenol Lab.). It was given i.v. in a dose of 1 [illegible] μg/kg b.w.

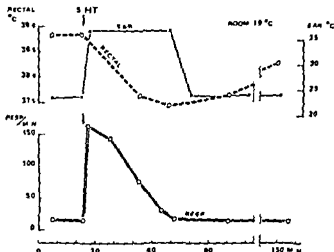


Fig 2 Effects of intraventricular injection of 5-hydroxytryptamine (5-HT) in goat A
Rapid onset of peripheral vasodilatation (ear temp) and of polypnea caused a marked fall in rectal temperature
Dose of 5 HT = 12 5 µg/kg b w

Rectal temperature was measured at intervals with a mercury thermometer and blood temperature was obtained by use of a thermo-couple catheter inserted into the right auricle of the heart Brain temperature was recorded by a thermo-couple needle permanently implanted laterally in the forebrain (goat A) or in the preoptic region (goat B) An index of peripheral blood flow was obtained by thermo-couple recordings of ear surface temperature Room temperature was in all experiments maintained between 18 and 20° C

Results

Site of injection

The position of the cannulae in the 3 goats is shown in Fig 1 As seen in this figure the injections in goat A (having 2 cannulae) could either be made ventrally in the anterior part of the 3rd ventricle, or in its posterior part In goat B the injections were made dorsally in the anterior part of the ventricle and in goat C in the posterior part at approximately the same site as injections performed *via* the posterior cannulae in goat A

Effects of 5 HT in goats A and B

The doses of 5 HT injected into goats A and B were 2 5, 5 and 12 5 µg/kg b w The higher dose was used in most experiments So far 9 expts involving intraventricular injection of 5 HT have been made in goat A and 7 in goat B In these 2 animals the injections invariably caused peripheral vasodilatation, polypnea, increased sweating on the nostrils and a gradual fall in rectal temperature (Fig 2) Minimal rectal temperature (about 1° C below preinjection level) was recorded in about 40 min Brain and blood temperature fell more rapidly and reached minimum level within 20 min Polypnea became apparent within a minute when the injections were performed in the anterior part of the 3rd ventricle and within 2 min when performed via the posterior cannula in goat A Respiratory frequency reached its maximum within 5 min but remained above normal for 20 to 40 min Peripheral vasodilatation was always seen to outlast the polypnea Within 2 hrs after the injection of 5-HT the body temperature was back in the normal range

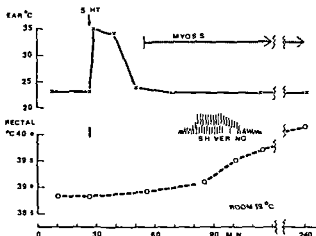


Fig 3 *Delayed fever response to intraventricular 5-HT in goat C* This animal later developed encephalitis For further details see text Dose of 5-HT = 12 5 μg/kg b w

Effects of 5 HT in goat C

The first intraventricular injection of 5 HT (12 5 μg/kg b w) into goat C was made on the 8th postoperative day, i e sooner after operation than in the other two goats Here the response (Fig 3) differed greatly from that obtained in goats A and B Polypnea did not occur, and the initial period of peripheral vasodilatation lasted only 10 min At this stage the animal started to shiver and shivering became intense 50 min after the injection of 5 HT During the period of shivering rectal temperature rose steeply by about 1° C Although shivering gradually disappeared, the rectal temperature remained at febrile level when measured 4 hrs after the injection The following morning it was normal again

The same intraventricular dose of 5 HT was given to goat C on the 11th and on the 14th postoperative days On both occasions the injection was followed by peripheral vasodilatation lasting for about half an hour During the period of peripheral vasodilatation the rectal temperature fell by 0 5° C Then peripheral vasoconstriction reappeared and myosis developed concomitantly with a slow rise in rectal temperature No shivering was observed, however, and rectal temperature did not exceed preinjection level until 3 hrs after the 5 HT had been given It continued to rise for another 2 hrs to reach a final level of 0 6° C above preinjection temperature

In the morning of the 19th postoperative day goat C was found to have marked myosis and a rectal temperature 1 5° C above normal Symptoms of encephalitis developed and for this reason the goat was sacrificed on the 21st postoperative day

The effects of intraventricular 5 HT in goat C especially after the first injection were very similar to those seen after intravenous injections of exogenous pyrogen in this species (delayed onset of myosis of shivering and of temperature rise) (Andersen Andersson and Gale 1962) Since goat C later on developed encephalitis it was thought that the intraventricular administration of 5 HT in this animal either might have caused a release of endogenous pyrogen from a developing inflammatory process

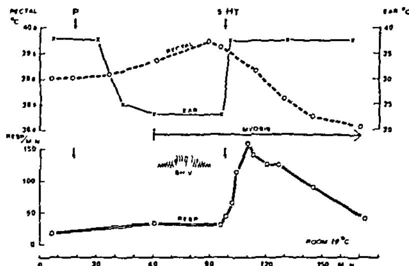


Fig. 4. *Effects of intraventricular 5-HT in goat A during fever induced by exogenous pyrogen.*
P = intravenous injection of pyrogen (pseudomonas polysaccharide 1.2 µg/kg b.w.).
Dose of 5-HT = 12.5 µg/kg b.w.

in the brain, or might have potentiated endogenous pyrogen present in subthreshold amounts. To test the latter possibility, the effects of 5-HT were studied in goats A and B after pretreatment with exogenous pyrogen.

Effects of 5-HT in the pyrogen treated animal

Intraventricular injections of 5-HT (12.5 µg/kg b.w.) were performed after previous intravenous administration of pyrogen in goat A and B. Also now 5-HT caused a mobilization of heat loss mechanisms and a considerable drop in body temperature, although the polypnea was less pronounced and somewhat delayed. As regards peripheral vasodilatation, the response was dependent of the stage of pyrogen fever at which the intraventricular injection of 5-HT was performed. When injected 80 min after the administration of pyrogen (when the fever had passed maximum) the onset of peripheral vasodilatation was as rapid as in the animal not treated with pyrogen (Fig. 4). On the other hand, when the injection of 5-HT was made 50 min after pyrogen had been given (during the rising phase of fever) peripheral vasodilatation appeared first after a latency of 10 min. The fall in body temperature was delayed correspondingly.

Catecholamines

When adrenaline or noradrenaline (0.3 and 0.7 µg/kg b.w.) were injected into the 3rd ventricle of the goats, no significant change in rectal temperature was seen during the following 2 hrs. Only the higher dose of noradrenaline caused a slight increase in the respiratory rate after 5 to 10 min. This moderate polypnea was not accompanied by apparent peripheral vasodilatation.

Discussion

In homeothermic animals body temperature seems to be regulated to a great extent by the activity of thermo-sensitive neurons located in the rostral hypothalamus (cf Euler 1961) Recent micro-electrode studies (Hardy, Hellon and Sutherland 1964) have demonstrated the presence of two kinds of thermo sensitive neurons in this part of the brain, i e some reacting specifically when the hypothalamic temperature is raised ("hyperthermia detectors") and other reacting when hypothalamic temperature is lowered ("hypothermia detectors")

It has been shown that local displacement of the temperature of the rostral hypothalamus not only affects neuro-muscular, but also hormonal mechanisms of importance in temperature control (cf Andersson *et al* 1964) Local cooling of the "thermoregulatory center" in the rostral hypothalamus causes thyroid activation and increased release of sympathic amines, whereas the corresponding warming blocks the thyroid and sympathico-adrenomedullary activation normally occurring during a general cold stress The increased release of adrenaline and noradrenaline during local cooling of the rostral hypothalamus occurs in the adrenal medulla and at peripheral nerve endings, and the blood brain barrier may prevent central effects of the rised catecholamine level of the blood To what extent and in which manner a changed hypothalamic temperature affects central release of biogenic amines is not known As mentioned above, Feldberg and Mayers (cf Feldberg 1965) have suggested that local levels of 5-HT and catecholamines have opposite effects on the hypothalamic "thermoregulatory center" An increased hypothalamic release of 5-HT should rise body temperature and the corresponding release of catecholamines should lower body temperature From the present experiments it appears evident that such is not the case in the goat Moderate amounts of catecholamines injected into the 3rd brain ventricle did not cause any significant change in the body temperature in this species In 2 apparently healthy animals intraventricular injections of 5-HT caused the mobilization of heat loss mechanisms and a fall in body temperature A similar response to intraventricular 5-HT has recently been observed in the rabbit (Cooper, Cranston and Honour 1965) The discrepancy between these results and those obtained in the cat (*cf* Feldberg 1965) could then be explained on the basis of species differences

Since the latency time for the response in the goat was somewhat longer when the injections were performed in the posterior as opposed to the anterior part of the ventricle, it appears that 5-HT somehow stimulated the "hyperthermia detectors' in the anterior hypothalamus Although the dose of 5-HT per kg b w was lower than that generally used in similar experiments in the cat (*cf* Feldberg 1965), they were still so high that it is questionable whether the local concentration of 5-HT could reach such levels under physiological conditions

Still to be explained is the febrile response obtained in goat C after intraventricular injections of 5 HT ——— a response similar to that reported to occur in the cat (*cf* Feldberg 1965) The posterior site of injection in this goat does not seem to

account for the different response observed, since in goat A, stimulation of the heat loss mechanisms was obtained after injections of 5-HT both in the anterior and the posterior part of the 3rd ventricle On the other hand, goat C showed symptoms of a brain infection on the 19th postoperative day, and it is likely that a latent inflammatory process was already present in the brain when the intraventricular injections of 5 HT were performed In such a case the injection of 5-HT into the ventricular system might have caused a release of pyrogen from the inflammatory process, leading to a delayed and sustained febrile response That 5-HT should have potentiated pyrogen already released from the inflammatory process seems unlikely, since the substance still caused heat loss reactions and a temperature drop in the 2 apparently healthy goats after pretreatment with pyrogen Swank and Hissen (1964) have shown that intracarotid injections of 5-HT increase cerebral circulation in the dog and may cause increased permeability in the brain It is not unlikely that such circulatory changes would release pyrogen from a brain inflammatory process This may be the explanation for the febrile response to 5-HT observed in goat C

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Connections between Adrenergic Nerves and other Tissue Components in the Eye

By

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Abstract

EHINGER, B *Connections between adrenergic nerves and other tissue components in the eye.* Acta physiol scand 1966 *67.* 57—64.

The connections between adrenergic nerve fibres and other ocular structures were studied in normal embryonic material (man, dogs, cats, guinea pigs, and rats) as well as with a special vessel injection technique (adult rats, guinea pigs, and rabbits) It was established that adrenergic fibres are a normal

able in that they seemed to possess adrenergic fibres. No adrenergic innervation to the melanophores was apparent

Recent results obtainted with the fluorescence technique of Falck and Hillarp (*cf* Falck and Owman 1965) regarding the distribution of adrenergic nerves to the eye (Ehinger 1964 a and b, 1966 a—c, Laties and Jacobowitz 1964, Malmfors 1965) have rendered questionable many of the classical concepts about the distribution of adrenergic fibres Such fibres were found to occur in several parts of the eye where they were not expected from classical physiological and pharmacological work, which has not recognized adrenergic nerve terminals in tissue lacking nerve cells, secretory cells or muscle cells So far, however, it has not been possible to assess accurately to what extent the demonstrated adrenergic nerves were associated with small vessels, a question of obvious importance for the physiologist With a vascular injection technique and special studies on embryonic and adult tissue, the connections between adrenergic nerves and vessels and some other structures in the eye have now been studied

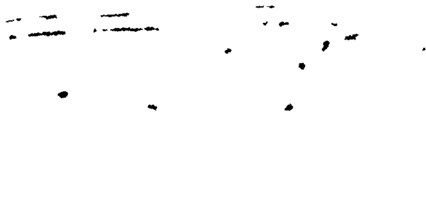


Fig 1 Embryonic cornea, guinea pig 62 days Subepithelially the fluorescent adrenergic fibres form a close meshed onelayered lattice, from which twigs issue into the epithelium Dark field fluorescence micrograph × 150

Materials and methods

Eyes were taken from 11 human embryos obtained by surgery The crown heel lengths of the foetuses varied between 12 and 32 cm corresponding to the 15th and 26th week of gestation respectively Eyes were also taken from the foetuses of 5 rats 5 guinea pigs, 2 dogs and 6 cats at approximately full term and from new born animals up to several weeks of age

To demonstrate the blood vessels injections were made through the carotids of adult rats guinea pigs and rabbits 5 of each The animals were killed with an overdose of ether or pentobarbital sodium and the vascular tree was immediately washed out with 6 % dextrane containing 0 9 % sodium chloride and a few drops of amyl nitrite per 100 ml solution. Indian ink containing 6 g dextrane (MW approx. 80 000) 0 9 g sodium chloride and a few drops of amyl nitrite per 100 ml was then injected at a pressure of about 250 mm Hg and at 37° C The injection was continued for 3—5 min until the iris appeared almost black With this pressure and the vasodilatation produced by amyl nitrite complete filling of all parts of the vascular tree was obtained as secured by the examination of sections stained in hematoxylin and eosin Whole eyes or appropriate pieces of tissue were excised and processed as has been described (Ehinger 1964 b) for the demonstration of the adrenergic fibres (*cf* Falck and Owman 1965) The vessels and nerve fibres were followed in serial sections The micrographs of the injected specimens were taken with a bright field condenser in the fluorescence microscope in order to show the injected vessels

Results

Embryonic tissues The embryonic corneae contained a moderate number of adrenergic fluorescent nerve fibres, mainly situated in the frontal part of the corneal stroma (Fig 1), although not forming any distinct layer except just beneath the epithelium (*cf* below) In no case were the adrenergic fibres seen together with any vessel As in adult tissues some fibres looked like a string of beads, but the intercalated segments had a much more prominent fluorescence in the embryo Furthermore, there were many smooth fluorescent fibres, which also showed a higher fluorescence than is normal in the preterminal fibres of the adult animal The fibre density of the apex corneae was not significantly different from that of more peripheral parts The limbus had a rather high density of adrenergic fibres, partly forming the usual vascular sheath and partly running freely in the stroma The overall number of corneal fibres was least in the human embryo, where they did not appear until the

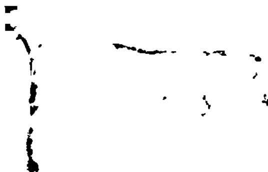


Fig 2 Tangential section through the surface layers of the cornea in a new born dog Above and to the left there is a nerve trunk, slightly out of focus but it can still be seen that it contains several beaded axons Some repeatedly branching varicose single terminal axons issue from the trunk. Dark field fluorescence micrograph, × 350

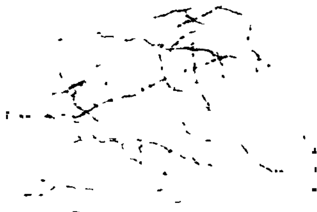


Fig 3 Adrenergic network superficially in the limbus region guinea pig Clearly the majority of the fibres are non vascular Dark field fluorescence micrograph × 240

24 cm stage At this stage and in all the animal foetuses studied, adrenergic fibres could be seen intraepithelially in the cornea (Fig 1) These fibres seemed to originate from a rather close meshed plexus of fine fibres just under the epithelium The number of intraepithelial and juxtaepithelial fibres was considerable just before and immediately after birth The number evidently decreases shortly afterwards, since in animals only one or two weeks old few fibres remain intraepithelially and juxta epithelially, and in the adult animals of all species, no fibres are to be found at these sites

The ramifications of a single adrenergic fibre can be studied excellently in the corneal epithelium of a new born animal Smooth preterminal fibres were often seen to change into varicose fibres within the nerve trunk before dividing into a large number of branches, which form a tree like structure (Fig 2 There were no anastomoses visible, nor were there any specialized end-organs

Adult tissues The injection experiments sometimes induced a slightly increased degree of autofluorescence in the tissues, but did not appreciably impair the demonstrability

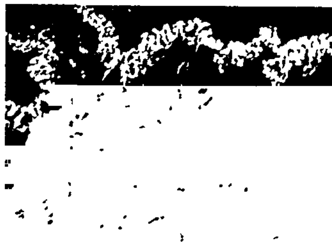


Fig 4 Iris rabbit vessels injected with Indian ink At the top the dense adrenergic plexus of the dilator The majority of the adrenergic fibres in the iris stroma are non vascular Bright field fluorescence micrograph × 190

of the adrenergic fibres Thus, for instance, the adrenergic fibres of the retina remained intact, in spite of the fact that the fluorescence of these fibres is very easily lost if the specimen is not properly handled Further, the overall distribution of the adrenergic fibres did not differ from the normal pattern The beaded appearance of the terminal fibre may, however, be less distinct

In the cornea proper, the adrenergic fibres were never observed to follow any vessels As seen in the phase-contrast microscope, the adrenergic fibres very often run in a bundle of thick, non fluorescent nerve fibres The overall distribution of the adrenergic fibres has been described previously (Ehinger 1964 a and b 1966 a—c) In the limbus region, some fibres followed the vessels, mainly the arteries, but a considerable number also ran freely in the stroma, as can readily be seen even in non injected specimens (Fig 3) The abundant adrenergic fibres of the chamber angle of the guinea pig (Ehinger 1964 b) were very rarely connected to any vessels, whereas those of the rabbit were always associated with a vessel when occurring in any significant number However single varicose adrenergic fibres without any connection to vessels could occasionally be observed in the rat and the rabbit, at times in the pectinate ligament The aqueous drainage system in the sclera could be identified since it did not fill with carbon particles, at least not its inner parts In the rat and rabbit, adrenergic fibres occurred at the walls of the aqueous drainage system of the sclera in small to moderate amounts the least at its innermost parts

In the iris of all species studied there was a plexus of varicose adrenergic fibres running freely in the iris stroma without any connection to vessels (Fig 4) The fibres were more densely arranged in the frontal parts than in the others No connections were obvious between the adrenergic fibres and the spider shaped, non fluorescent melanophores in either the rabbit, the guinea pig or the dog In these



Fig 5 Semitangential section through the epithelium of the ciliary body Rabbit, vessels injected Only a minor part of the adrenergic fluorescent fibres are associated with the vessels Bright field fluorescence micrograph, × 250

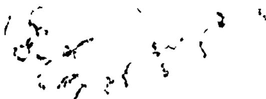


Fig 6 Ciliary processes, rabbit, vessels injected Some of the fluorescent adrenergic fibres surround the somewhat dilated vessels In the tangentially cut part of the process to the right, fluorescent fibres run separated from the vessels Serial sections proved that these fibres to a considerable extent ran without vascular connections Bright field fluorescence micrograph, × 210

species there was a considerable accumulation of melanophores in the limbus region and in the ciliary body without any corresponding accumulation of adrenergic nerves It cannot be excluded that some of the melanophores of the medial and frontal thirds of the iris may be associated with the varicose adrenergic nerves, such as seems to occur in the cynomolgus monkey (Ehinger 1966 b) However, no differences such as occur in the monkey were evident in the autofluorescence characteristics of the melanophores of the iris and those of the rest of the uvea in the rabbit, the dog, or the guinea pig

In the sphincter, some of the adrenergic nerves present were evidently associated with vessels (mainly small arterioles) especially in the peripheral parts, but the majority of the adrenergic fibres had no vascular connection

In the ciliary body, the adrenergic varicose nerves of the ciliary muscle of the guinea pig ran without vascular connections The subepithelial plexus found in all species was only in places directly associated with any vessels (Fig 5), which

mainly are veins in this region In the ciliary processes, the adrenergic varicose nerves could be seen to form one plexus around the majority of the vessels, and another with varying density in connection with the epithelium (Fig 6) There are many interconnections between these two systems, which could not always be separated completely Some but not all of the small veins were entirely without fluorescent fibres The plexus at the epithelium was more obvious in the rabbit than in the other species, and was especially seen at the bases of the processes and at their connection with the iris

Discussion

It is well known that keratitis may cause an ingrowth of vessels into the cornea Such vessels possess adrenergic nerves, capillaries excepted, and possibly the corneal adrenergic fibres observed are remnants of vessels which have been obliterated However, the facts that no cornea has been shown to lack the adrenergic fibres, that they hardly ever follow blood vessels, that they seem mostly to follow nerve trunks, that they occur in the outer layers of the cornea only (ingrown vessels can generally be expected to lie throughout the stroma), and that they occur even more abundantly in embryonic than in adult corneae demonstrate that they are a normal constituent of the cornea

The injection experiments clearly indicate that the majority of the adrenergic fibres in the sphincter (*cf* Ehinger 1964 a and b, 1966 a—c Laties and Jacobowitz 1964, Malmfors 1965) do not belong to the blood vessels Pharmacologically, sympathomimetics as well as sympathetic blocking agents have been shown to affect the sphincter, establishing further the adrenergic influence in the region (Schaeppi and Koella 1964, Tákáts 1964) Since the cholinergic parasympathetic innervation in the sphincter is well established, there are now strong reasons for regarding the sphincter pupillae as doubly innervated A similar double innervation has been shown also in the dilator (Ehinger and Falck 1965)

The distribution of nerves at the ciliary epithelium both in the ciliary body proper and the ciliary processes fully corroborates earlier results (Ehinger 1964 b) and favours the presumption of an adrenergic innervation of the epithelium There is pharmacological and physiological evidence of an influence from the sympathetic nervous system and sympathomimetics on the secretion of aqueous humour (Weekers *et al* 1955, Swegmark 1963 Berggren 1965) However the capillaries of the processes also have a unique supply of adrenergic fibres which clearly separates them from most capillaries of connective glandular and fat tissue of the orbit (Ehinger, unpubl observ) and muscular tissue (Fuxe and Sedvall 1965) Thus, not only the diameter of the vessels and the ultramicroscopic structure (Taniguchi 1962) of the wall but also their supply of adrenergic nerve fibres distinguish these vessels from ordinary capillaries It should be noted that the effect of sympathomimetics on the production of aqueous humour may be due to an adrenergic innervation of these capillaries or of the ciliary epithelium or of both

The adrenergic fibres running freely in the connective tissue in the limbus,

in the cornea, and in the iris could not be expected judging from the results of classical physiology and pharmacology The function of the vegetative nervous system is generally assocaited with the regulation of more specialized target organs such as secretory cells or muscle cells The presence in the mentioned regions of adrenergic nerve terminals originating from — or passing through — the superior cervical ganglion (Ehinger 1964 b, 1966 a) indicates that there may be a sympathetic innervation of some constituent of connective tissue It could be presumed that some of the varicose adrenergic fibres possibly represent fibres which attain their rosary like shape already when passing through the connective tissue before reaching their effector cells However, this is not conceivable considering the well developed plexus of e g the limbus, which shows no observable uniformity in the pattern of fibre directions such as could be expected were they all intended for e g blood vessels

Cholinesterase-containing fibres have been demonstrated running freely in the connective tissue to the same extent as the adrenergic fibres discussed above (Ehinger 1966 d), and a double innervation of a connective tissue component is consequently possible In the dilator region of the rat iris (Ehinger and Falck 1965) and possibly also in some other muscular tissues (Jacobowitz and Koelle 1965) adrenergic and cholinesterase containing fibres run together It remains to be shown to what extent this is the case in connective tissues also

In the iris of the cynomolgus monkey, the distribution of the adrenergic fibres suggests that a special kind of melanophores with a red autofluorescence here represent one kind of stromal receptors (Ehinger 1966 b) It cannot be excluded that in lower animals there is also an innervation of some of the melanophores, particularly in the iris However, most of them must be regarded as having no such innervation (*cf* Ehinger 1964 b) since an increase in their number is not invariably followed by an increased number of adrenergic fibres

The intraepithelial adrenergic corneal fibres are of special interest and importance First, the pattern of adrenergic fibres agrees with Hillarp's principle of convergence and the multiple distribution of the end twigs in the single neuron (Hillarp 1959) A single nerve fibre branches richly at its target organ so that many cells are innervated by one nerve fibre Conforming results have also been obtained in many other organs (*cf* Falck 1962, Norberg and Hamberger 1964, Falck and Owman 1965, and Malmfors 1965) The second point of interest regarding the corneal innervation lies in the fact that there has so far been no reports of adrenergic fibres, which are only present during a restricted period of the development such as happens with the adrenergic fibres in and at the corneal epithelium At present it is not possible to interpret the function of this innervation There are no indications of any secretory or muscular function of the corneal epithelium at any stage of development (*cf* Mann 1950) An influence on the mitotic activity of sympathomimetics has, however, been found in the corneal epithelium Friedenwald and Buschke 1944) as well as in tissue cultures Barka 1964) It follows that there is a possibility that the adrenergic fibres in connective tissue, which have as yet no known and well-defined target organ might regulate the state of metabolic activity of the tissues

mainly are veins in this region In the ciliary processes, the adrenergic varicose nerves could be seen to form one plexus around the majority of the vessels and another with varying density in connection with the epithelium (Fig 6) There are many interconnections between these two systems, which could not always be separated completely Some but not all of the small veins were entirely without fluorescent fibres The plexus at the epithelium was more obvious in the rabbit than in the other species, and was especially seen at the bases of the processes and at their connection with the iris

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The adrenergic fibres running freely in the connective tissue in the limbus

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Studies on the Stimulating Effects of Adrenaline and Noradrenaline on Respiration in Man

By

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Abstract

LUNDHOLM L and N SVEDMYR *Studies on the stimulating effects of adrenaline and noradrenaline on respiration in man* Acta physiol scand 1966 67, 65—75

Adrenaline infused intravenously at a rate of 0 15 µg kg min increased the ventilation and CO_2-elimination by a maximum of 45 % in experiment on man. Since adrenaline increased the CO_2 tension and decreased the pH in the plasma of arterial blood, the respiratory increase after adrenaline was assumed to depend, at least in part, on an increased CO_2-production of metabolic origin About half of the increase in CO_2-production was estimated to be due to raised oxygen consumption and the [illegible]

The effects of adrenaline and noradrenaline on the respiration are twofold In lower doses the respiration is stimulated, while higher doses produce depression — adrenaline apnoea In this investigation we have studied the stimulatory effect of adrenaline and noradrenaline on the respiration in man and have attempted to localize the mechanism responsible for these effects

Griffith, Emery and Lockwood (1940) and Young (1957) studied the respiration-stimulating effect of adrenaline in the cat and found some relationship between stimulation of respiration, metabolism and lactic acid production

Whelan and Young (1953) found in experiments on man that adrenaline had a stronger and more protracted stimulatory effect on the respiration than noradrenaline Both drugs reduced the alveolar pCO_2 Bradley *et al* (1954) found in man that adrenaline in a dose of 0 20 µg kg min stimulated the respiration and decreased the pCO_2 and increased the pH of arterial blood Coles *et al* (1956) studied the question of whether adrenaline and noradrenaline had any central point of attack on the respiration They infused the catecholamines into the carotid or vertebral

artery in man but observed no stimulative effect on the respiration Barcroft *et al* (1957) found that the stimulative effect of noradrenaline on the respiration was prolonged and accentuated when the CO_2 content of the inspired air was raised They discussed the possibility that noradrenaline sensitized some receptors for CO_2, but on analysing their values they found no support for this hypothesis Finally, Cunningham, Lloyd and Patrick (1963) found that the respiration stimulating effect of noradrenaline was eliminated if the partial pressure of O_2 in the inspired air was increased, and they considered the possibility that noradrenaline sensitized O_2-sensitive receptors

Our principal aim was to investigate the possible significance of increased metabolism and lactic acid production for the stimulative effect of the catecholamines on the respiration in man We therefore studied the effects of adrenaline and noradrenaline on the O_2 consumption, CO_2 production and respiratory volume and frequency, and also on the pCO_2, pH, bicarbonate concentration and NA^+ and K^+ concentrations of the arterial plasma and the lactic acid concentration of the arterial blood

Methods

The experiments with adrenaline were performed on 7 healthy male subjects of ages 22—30 years, and those with noradrenaline on 4 other subjects The subjects came to the laboratory in the morning having fasted since the previous evening and lay on a couch The brachial artery was punctured at the cubital fossa of one arm and a fine teflon catheter was introduced through the puncture needle which was then withdrawn This catheter was used for recording the arterial blood pressure by means of a Statham pressure transducer and a Grass polygraph and also for the withdrawal of blood samples An Olofson needle was introduced into a subcutaneous vein in the other arm and 1 ml 5 % Heparin^R was then injected Through this needle a continuous infusion of either physiological saline solution of adrenaline or noradrenaline solution was given at a rate of 0 2 ml min by means of an infusion apparatus The pulse rate was recorded with a Grass FCG tachygraph and the O_2 consumption CO_2 production and respiratory volume were recorded continuously with a Hartman and Braun metabolic recorder This apparatus made continuous measurements of the O_2 and CO_2 contents of the expired air and of the respiratory volume and frequency All these parameters were recorded on a Grass polygraph From the product of the ventilation/min and O_2 or CO_2 difference between the inspired and expired air the O_2 consumption or CO_2 production could be recorded. Because of the lag of the O_2 and CO_2 recording time periods shorter than 2 min were not measured

When the catheters had been introduced and the subject had rested for about 60 min the O_2 consumption CO_2 production and respiratory volume and frequency were determined over two basal 10 min periods A basal sample of arterial blood was then taken for lactic acid determination (in whole blood) and with a paraffined syringe further blood samples were taken for determination of the plasma pCO_2 pH standard bicarbonate at 37 C and Na and K concentrations The blood was centrifuged immediately under paraffin oil at 300 × g and the plasma was drawn into paraffined syringes where it was kept until the analyses were performed L-adrenaline of L-anoradrenaline was then infused i v for 30 min The catecholamines were dissolved in 0 9 % saline solution to which was added

It was found however (Table II) that when the arterial blood was analyzed with this method there was an increase of the pH and a decrease of pCO_2 of blood which remained for 30 min after the end of

TABLE I The influence of adrenaline (0.15 $\mu g/kg/min$ for 30 min) on the respiration, circulation and metabolism of man Mean ± S.E.M. of 7 tests FFA (=free fatty acids) values from Svedmyr (1966)

Physiological function	Basal values	Change during infusion of adrenaline — Time of infusion min: 3	8	30
Ventilation l/min	5.5±0.28	1.6±0.53 P<0.05	2.3±0.41 P<0.01	2.3±0.43 P<0.01
Respiratory rate/min	9.9±0.8	1.1±0.5	1.1±0.5	2.1±0.7 P<0.02
O_2 consumption ml/min	272.3±9.3	48.1±8.5 P<0.01	60.7±8.0 P<0.001	93.1±15.2 P<0.001
CO_2 production ml/min	209±9.4	73.3±21.2 P<0.02	105.9±11.5 P<0.001	92.7±7.9 P<0.001
Pulse/rate/min	55±3.2	13.3±3.6 P<0.02	16.9±3.6 P<0.01	23.0±5.3 P<0.01
Systolic B.P. mm Hg	115.8±3.2	−2.6±4.6	19.1±3.7 P<0.01	24.1±5.2 P<0.01
Diastolic B.P. mm Hg	55.0±1.8	−4.1±3.3	−5.4±1.6 P<0.02	−7.0±2.3 P<0.05
Lactic acid in blood meq/l	0.53±0.03	0.03±0.04	0.29±0.08 P<0.02	0.54±0.15 P<0.001
Blood plasma				
Na^+ meq/l	137.6±1.6	1.1±0.5 P<0.05	1.5±0.8	2.7±0.8 P<0.02
K^+ meq/l	3.96±0.14	−0.02±0.04	−0.41±0.1 P<0.05	−0.98±0.11 P<0.001
Standard bicarbonate meq/l	17.96±0.490	0.07±0.23	−0.49±0.19 P<0.05	−1.50±0.27 P<0.01
pH	7.320±0.017	−0.005±0.005	−0.016±0.006 P<0.05	−0.037±0.006 P<0.001
FFA meq/l (n=5)	0.69±0.08	–	0.32±0.12 P<0.05	0.77±0.16 P<0.01
pCO_2 mm Hg	35.6±0.68	1.21±0.54 P<0.05	1.10±0.51	0.66±0.73

the adrenaline infusion As this after effect was an unprobable phenomenon we studied the method for determining pCO_2 in whole blood more closely and found that if the blood sample was stored in [illegible]

As we had no opportunity to determined pH and pCO of the blood immideately after the sampling (3 samples wer taken within 10 min) we decided instead to examine the pH and pCO_2 of pl

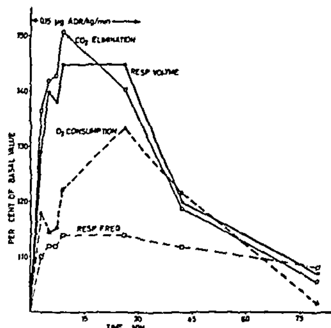


Fig 1 Effect of adrenaline infusion on the respiratory minute volume CO_2 elimination O_2 consumption and respiratory frequency in percent of the basal value Mean of 7 experiments

according to Astrup (1956) We made some control experiments and found that plasma could be stored in paraffined all glass syringes for 4 hrs without change of pH or pCO_2

Lactic acid in 1 ml whole blood was determined enzymatically according to the method of Lundholm Mohme Lundholm and Vamos (1963) The K and Na concentrations in 2 ml plasma were determined by means of a Perkins and Elmer flame spectrophotometer

Some values on the influence of the catecholamines on the free fatty acids (FFA) in plasma have been *included in Table I and III These values have been taken from Svedmyr (1966) These experiments* were performed as described above but on other subjects FFA was determined according to Trout Estes and Friedberg (1960

Results

Adrenaline The effect of adrenaline given for 30 min at a rate of 0 15 μg/kg/min on the ventilation and respiratory frequency is illustrated in Fig 1 and Table I The ventilation increased rapidly being approximately 40 % higher after about 5 min the increased values persisted throughout the infusion and then began to decline, regaining the original value after 90 min The increase in respiratory frequency was less than that of the ventilation indicating that the respiratory volume had also increased The CO_2 production also increased, and the curves for the CO_2 elimination and the ventilation were very similar The O_2 consumption also increased *but* this increase during the infusion period was percentually considerably smaller than for the CO_2 elimination

The increase in CO_2 elimination could either have been caused by an actual increase in CO_2 production in the tissues or by the washing out of CO_2 due to respiratory stimulation It may be seen in Fig 2 that the pCO_2 in the plasma of arterial blood increased during the adrenaline infusion while at the same time the pH decreased This can hardly be interpreted in any other way than that the increase in the CO_2 elimination was due to increased CO_2production Lundholm and Sved-

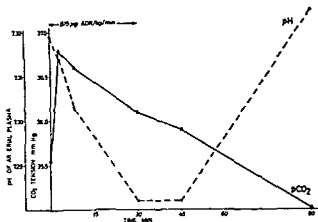


Fig 2 Effect of adrenaline infusion on the pH and pCO_2 in plasma from arterial blood

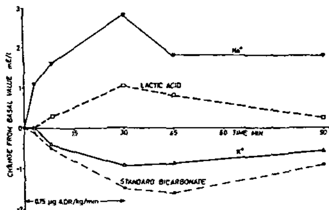


Fig 3 Effect of adrenaline on the Na , K and bicarbonate concentrations of the plasma from arterial blood and on the lactic acid concentration of whole blood

myr (1965) also found that the CO_2 production from the forearm musculature increased on adrenaline infusion simultaneously with a rise in pCO_2 in the arterial blood and the venous blood from the musculature

Adrenaline also influenced the acid base equilibrium in the blood (Fig 3) Both the lactic acid content of the blood and the plasma Na^+ and FFA concentration increased On the other hand the plasma K_+ concentration decreased, as also did the plasma standard bicarbonate concentration

The effect of adrenaline infusion on the pulse and blood pressure is shown in Table I The pulse rate increased markedly, the systolic blood pressure rose and diastolic fell During the first minute both the systolic and diastolic blood pressure fell, however, so that a true depressive effect occurred

In another series of experiments the influence of adrenaline (0 1 µg/kg/min for 30 min) was studied in man In this series the pH and pCO_2 was measured on samples of whole arterial blood which had been stored in iced water for 1—3 hrs before the analysis The effect of adrenaline on respiration was principally the same as in

TABLE II The influence of adrenaline (0 1 $\mu g/kg/min$) on pH and pCO_2 in mm Hg of iced whole arterial blood Mean of 4 tests

	pH	pCO_2
Basal values	7 349±0 027	35 4±1 6
Change from basal values		
Infusion of adrenaline		
3 min	0 033±0 015	-11 9±2 7 P< 0 02
8 min	0 035±0 017	-10 4±3 5 P< 0 05
30 min	0 012±0 011	-11 9±2 4 P< 0 02
Post infusion period		
45	0 015±0 014	-10 7±3 4 P< 0 05
60	0 024±0 019	-9 33±4 2

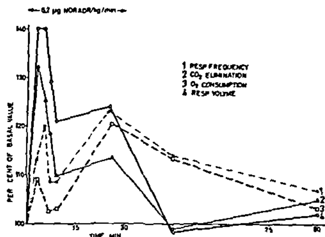


Fig 4 Effect of noradrenaline infusion on the respiratory minute volume CO_2 elimination, O_2 consumption and respiratory frequency in experiments on man Mean of 4 experiments The values are given as per cent of the basal values

the tests with 0 15 μg adrenaline/kg/min The changes of pH and pCO_2 in the blood were however quite different as pH increased and pCO_2 decreased (Table II) These effects remained after the end of adrenaline infusion These results were in agreement with those of Bradley *et al* (1954) who also analyzed iced whole blood samples They infused however 0 2 μg adrenaline/kg/min As mentioned in 'Methods" determination of pCO_2 of iced whole blood samples may give unreliable results *Noradrenaline* infused i v at a rate of 0 2 $\mu g/kg/min$ stimulated the ventilation initially to a considerable degree (Fig 4 and Table II) The increase (40 %) was equally large during the first 5 min as after adrenaline, but the ventilation decreased despite

TABLE III The influence of L-noradrenaline (0 2 $\mu g/kg/min$ for 30 min) on the respiration circulation and metabolism of man Mean±S E M of 4 tests FFA values from Svedmyr (1966) In some cases the 3 and 8 min values have been summated in order to show significant effects

		Change during infusion of adrenaline		
		Time of infusion min		
Physiological function	Basal values	3	8	30
Ventilation l/min	6 2±0 3	2 6±0 7 P< 0 05	1 3±0 4 P< 0 05	1 5±0 4 P< 0 05
Respiratory rate/min	11 8±1 5	1 9±1 2	1 1±0 4	2 9±0 4 P< 0 02
O_2 consumption ml/min	258 0±9 2	19 7±6 1 P< 0 05	7 3±14 0	5[illegible] 3±6 1 P< 0 02
CO_2 production ml/min	215 0±3 5	68 0±16 2 P< 0 05	116 0±2 9 P< 0 01	28 5±10 1
Lactic acid in blood meq/l	0 77±0 29	0 1±0 04 P< 0 05	0 1±0 04 P< 0 05	0 07±0.20
Blood plasma				
pCO_2 mm Hg	34 8±0 73	–1 7±0 4 P< 0 05	0 07±1 5	–0 50±1 0
Standard bicarbonate meq/l	19 8±1 6	0 0±0 06	0 2±0 4	–0 7±0 5
pH	7 373±0 032	0 015±0 003 P< 0 01	0 015±0 003 P< 0 01	–0 010±0 016
FFA meq/l	0 79±0 03	–	0 30±0 06 P< 0 01	0 75±0 12 P< 0 01

continuation of the infusion, and regained the basal value considerably more rapidly than after adrenaline During the initial ventilation increase the CO_2 elimination was considerably raised, and even during the later period of the infusion there was some parallelism between ventilation and CO_2 elimination Apart from a transient initial increase the O_2 consumption did not rise to its maximal extent until the end of the infusion period

The increased CO_2 elimination after noradrenaline was combined with an initial decrease of the plasma pCO_2 and an increase in the pH At the end of the infusion, however, the increased ventilation and CO_2 elimination were combined with an almost unchanged pCO_2 and pH Noradrenaline caused an initial slight (but significant) increase of the lactic acid concentration of the blood The standard bicarbonate concentration decreased towards the end of the infusion period when the lactic acid concentration was normal Noradrenalin also increased the FFA content of plasma (Table III) The plasma Na and K concentrations were not affected

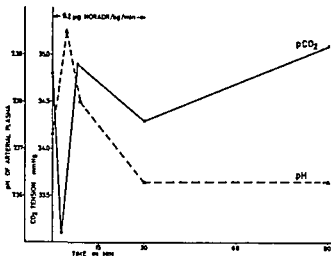


Fig 5 Effect of noradrenaline infusion on the pH and pCO_2 in plasma from arterial blood Mean of 4 experiments

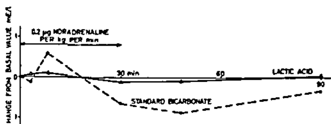


Fig 6 Effect of noradrenaline infusion on the lactic acid concentration of whole blood and plasma bicarbonate concentration

by noradrenaline The effects of noradrenaline on the blood chemistry were in agreement with the results of Lanthem *et al* (1957)

The pulse rate fell and both the systolic and diastolic blood pressure rose during infusion of noradrenaline

Discussion

Adrenaline There was a close relationship between increase in ventilation and CO_2 production in the experiment with adrenaline (Fig 1) The stimulating effect of adrenaline on the ventilation and CO_2 production was moreover increased in the same proportion after treatment with triiodothyronine (Svedmyr 1966) The pCO_2 *in the plasma of arterial blood increased by about 1 mm Hg after adrenaline* infusion, and the pH decreased Both these effects may result in respiratory stimulation (Perkins 1963, Kellogg 1964) Previous workers (Endres and Lucke 1925, Whelan and Young 1953) found that adrenaline reduced the alveolar pCO_2, and from these results concluded that the pCO_2 of the arterial blood would also be decreased The elevating effect of adrenaline on the plasma pCO_2 has been verified, however, in another investigation (Lundholm and Svedmyr 1965) It seems probable that the relationship between the pCO_2 of the arterial blood and the endtidal pCO_2 is not strictly correlated under these conditions Matell (1963) has also shown a difference between the endtidal pCO_2 and pCO_2 of the arterial blood during exercise The

results of Bradley *et al* (1954) are difficult to judge as these investigators analyzed iced samples of whole blood a method which may give erroneous results

If it is assumed that increased CO_2 production and pCO_2 and a reduced pH could explain, at least in part, the ventilatory increase after adrenaline, the next question is how the increase in the CO_2 production occurred This could be partly consequent to increased O_2 consumption, and partly induced by decreased alkali reserve, as a result of an increase in bound acids in the blood and tissues The total increase in O_2 consumption over the basal value during the adrenaline infusion (time 0—30 min) was on an average 1760 ml The corresponding increase in CO_2 production was 3120 ml During this infusion the plasma bicarbonate concentration decreased by 1 5 meq/l Assuming that this latter decrease also occurred intracellularly, i e in the water phase of the entire body — which is estimated as 70 % of the body weight — the total bicarbonate concentration of the body should have decreased by about 70 0 7 1.5 meq = 75 meq This corresponds to a CO_2 volume of 1600 ml If this calculation is correct, about half of the CO_2 production should therefore be due to increased acidity in the tissues and the other half to increased oxygen consumption

As shown in Table I the reduction in the plasma bicarbonate concentration could be explained, at least to a large part, by an increased lactic acid and FFA concentration in plasma and blood On the other hand adrenaline reduced the K^+ and raised the Na^+ concentration in the plasma Adrenaline also reduced the plasma phosphate content (De la Lande *et al* 1961) Noradrenaline reduced the standard bicarbonate without affecting the lactic acid concentration but raised the FFA concentration in plasma (Table III) It is therefore probable that FFA and lactic acid contributed to the reduction of standard bicarbonate after adrenaline and noradrenaline Other ions may also be of some importance It has been claimed (Bulbring 1962) that the catecholamines stimulate the Na^+—K^+—pump in the cell membrane The increase of Na^+ and decrease of K^+ in plasma after adrenaline may be explained in that way On the other hand noradrenaline had no definite effect of the Na^+ or K^+ content of plasma

Noradrenaline At first sight the respiration stimulating effect of noradrenaline appeared to be a somewhat weaker counterpart to that of adrenaline (*cf* Fig 1 and 4) In consideration of nature's sense of order it might be expected that the two catecholamines would have the same mode of action The initial phase of pronounced respiratory stimulation was combined however in the noradrenaline experiments with a reduction of the plasma pCO_2 and an increase of the plasma pH which appears to indicate that the increase in CO_2 elimination resulted from the washing out of CO_2 and not from increased CO_2 production In this respect there is probably a true difference between the effects of adrenaline and noradrenaline on the respiration By the end of the infusion period however when the O_2 consumption had increased, it was probable that a true increase of the CO_2 production had occurred and might contribute to the respiratory stimulation

The mechanism responsibe for the initial stimulating effect of the noradrenaline on the respiration is not clear. In some experiments, however, we have been able to confirm the findings of Cunningham, Lloyd and Patrick (1963) that the respiration-stimulating effect of noradrenaline was eliminated when the subject breathed pure O_2. This may indicate that noradrenaline influences O_2-sensitive chemoreceptors, perhaps by reducing the blood flow through the glomus caroticum. The adrenaline effect, on the other hand, persisted even when the subject breathed pure O_2. A quantitative analysis of the influence of O_2 inspiration on the stimulating effect of adrenaline on respiration should be made before it can be decided if adrenaline not in a higher dose influences O_2-sensitive chemoreceptors too.

The results indicate that in the tested dose adrenaline stimulates at least in part the respiration by means of an increase in CO_2 production and plasma pCO_2 and a decrease in the plasma pH. The noradrenaline effect seems at least in part to have a different mechanism, possibly associated with influence on O_2 sensitive receptors.

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Respiratory Enzyme Activities in Neurons and Glial Cells of the Hypoglossal Nucleus during Nerve Regeneration

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Abstract

HAMBERGER, A and J SJOSTRAND *Respiratory enzyme activities in neurons and glial cells of the hypoglossal nucleus during nerve regeneration* Acta physiol scand 1966 *67* 76—88

Respiratory enzyme activities were measured in the hypoglossal nucleus of the rabbit, 6, 14, 28,

reductase were analyzed histochemically Succinoxidase activity increased in single neurons up to the 14th postoperative day both on the unoperated and operated side and was on a high level until the 28th day A striking increase in NADH, tetrazolium reductase was demonstrated in the neurons on the operated side from the beginning of the second postoperative week In the micro-diver experiments the glial cells of the operated side revealed a significant increase in succinoxidase activity from the sixth to the 48th postoperative day The histochemical study of respiratory enzymes did not reveal any change in glial enzyme activity in the hypoglossal nucleus although morphological studies have shown hypertrophic astrocytes and reactive microglial cells in this region.

The importance of analyzing nerve and glial cells separately and to consider volume and mass changes in single cells is stressed

The amount of respiratory enzymes in nerve cell bodies after axon division has been the subject of inquiry in several studies over the years Different authors using biochemical or histochemical methods have given conflicting results Some authors have claimed an increase (Kreutzberg 1963, Fischer and Malik 1964, Harkönen 1964, Soderholm 1965) while others have reported a decrease in activity of neuronal respiratory enzymes (Howe and Flexner 1947, Friede 1959) during nerve regeneration The discrepancies between the different studies above may be due to differences in experimental material with respect to species, age, neuronal group, nerve cell survival or to the different biochemical and histochemical methods used

An important fact to be considered in biochemical studies is that in a given tissue sample, containing a neuronal group, the largest volume is generally occupied by glial cells The earlier concept that the glial cells only constitute a kind of supporting

tissue with negligible reactive changes during nerve regeneration (Howe and Flexner 1947) is today completely revised. Ultrastructural studies from the last years have revealed changes in mitochondria and other organelles (Andres 1961, Cervós-Navarro 1962) in the glia surrounding axotomized spinal ganglion cells. In addition, extensive proliferative and morphological changes have been described in the glial cells surrounding hypoglossal neurons during nerve regeneration (Sjöstrand 1965 a, b, 1966).

The aim of the present investigation is to evaluate the changes in enzyme activity of the nerve and glial cells during nerve regeneration. The two elements must be analyzed separately because the reaction may be entirely different in the two cell populations. The enzyme activity is analyzed on the single cell level with a quantitative technique and by histochemical methods. The results are compared with those obtained with biochemical methods on a macroscale. As material we have chosen the hypoglossal nucleus of the white rabbit in which the nerve cell changes (Brattgård *et al.* 1957) and the glial changes (Sjöstrand 1965 a, b, 1966) have been studied extensively during nerve regeneration.

Material and methods

Experimental material. 80 white rabbits weighing 1.5 to 1.6 kg were used. The right hypoglossal nerve was exposed under pentobarbital anesthesia and crushed at a point where it traverses the digastric muscle. This procedure was repeated twice with a forceps cooled to —70° in order to ensure complete destruction of the nerve. The animals were killed by an air embolus on various days from the 6th to the 48th day after nerve crush.

By a transverse cut through the middle of the hypoglossal nucleus the hypoglossal nuclei were divided into a rostral and caudal slice. The rostral slice was used for histochemistry and the caudal for microchemical determinations. In the biochemical experiments on a macroscale the hypoglossal nuclei were dissected and homogenized.

Determination of succinoxidase activity with the micro-diver technique (*Zeuthen 1953*). The caudal slice containing the hypoglossal nuclei was cut approximately 2 mm thick and placed in cold 0.25 M sucrose solution faintly stained with methylene blue which made the nerve cells slightly [illegible]

divers and the manometric measurement of the oxygen consumption was performed and calculated [illegible]

Incubation medium for succinoxidase determination: Na_2HPO_4–KH_2PO_4 buffer pH 7.4 37.5 mM, cytochrome c 8.6 × 10 [illegible] mM, Na succinate 25 mM, $AlCl_3$ 0.5 mM, $MgCl_2$ 0.5 mM (Slater 1949, Potter 1957).

The enzyme activity is expressed as 10 [illegible] µl O_2 per hr per [illegible] nerve cells and 10 [illegible] g dry weight of glial cells respectively.

Histochemical methods. For preparation of fresh, frozen sections the rostral part of the hypoglossal nuclei was covered with 10 per cent gelatin and immediately frozen in isopropane cooled with liquid nitrogen. Sections were cut in the cryostate (Dittes Duspiva) at —20° C with a nominal thickness of 14, 28 and 40 µ. Some material was fixed in chilled 4° C formalin of varying concentrations for different periods of time for the demonstration of formalin resistent oxidative enzymes (Walker

TABLE I Succinoxidase activity in neurons of the hypoglossal nucleus during axon regeneration Enzyme activity expressed as 10^{-4} µl O_2 per hr and 2 cells Mean values ± S E M.

Days after nerve crush	Operated side	Unoperated side
Controls	0 8±0 1 n=6	0 8±0 1 n=6
6	4 2±0 5** n=9	2 8±0 5* n=7
14	5 4±0 7** n=16	3 6±0 7* n=11
28	5 9±0 8**' ' n=10	3 4±0 7* n=8
48	4 0±0 5** n=6	2 6±0 6* n=6

* P<0 02 (from controls, i e unoperated animals).

** P<0 001 (from controls, i e unoperated animals)

' P<0 05 (from corresponding unoperated side of the operated animals)

and Seligman 1963) In a few experiments thin tissue slices were directly incubated in the medium Nitro-BT (Sigma Chemical company, St Louis) was used as final electron acceptor in all tetrazolium reductase experiments except a few, which were carried out with the MTT-cobalt method according to Thomas and Pearse (1961) All incubations were made at 37° C and the reactions were terminated in 10 % neutral formalin

Succinate tetrazolium reductase This enzyme was demonstrated according to Nachlas *et al* (1957) at pH 7 4 The period of incubation varied from 60 to 180 min

Cytochrome oxidase The method described by Burstone (1961) with 8-amino-1,2,3,4-tetrahydroquinoline (Eastman Kodak Rochester New York) as a coupler was used Incubation time was from 10 to 40 min This enzyme was only studied on the sixth and 14th day

$NADH_2$ tetrazolium reductase This enzyme, also called DPN-diaphorase, was demonstrated according to Friede *et al* (1963 with a method based on the principles given by Scarpelli *et al* (1958) using reduced diphosphopyridine nucleotide as substrate Incubation time varied from 20 to 60 min

Control experiments for the histochemistry As a control in all incubations some sections were incubated in medium lacking the substrate In cytochrome oxidase incubations the specificity of the reaction was tested by addition of potassium cyanide (Harkonen 1964) In a few experiments phenazine methosulfate (PMS) was added to the incubation medium for succinate tetrazolium reductase to a final concentration of 20—50 µg/ml (Harkonen 1964) The addition of PMS gave inconstant results For comparison some incubations were made with the MTT-cobalt method (Thomas and Pearse 1961) The MTT-cobalt technique was abandoned since the staining with this method gave a rather coarse precipitate with poor cytological location and results diverging from those obtained with the microdiver technique

Quantitative determination of succinoxidase activities in homogenates of the hypoglossal nucleus The right and left hypoglossal nuclei were cut out under stereomicroscope and weighed wet The tissue samples were then homogenized in small glass homogenizers and the homogenate added to Warburg flasks containing 2 5 ml of the succinoxidase incubation medium described under microdiver experiments The homogenates from two animals were pooled together Manometric readings were taken and the enzyme activity was expressed as µl O_2 per mg wet weight per hr All experiments were carried out at 37° C.

Results

Microdiver technique

Control material The results obtained from control rabbits are given in Table I and II, and plotted in Fig 1, 2 and 3 The mean value for succinoxidase activity was 0 8 ×

TABLE II Succinoxidase activity in neurons and glial cells of the hypoglossal nucleus during axon regeneration

Enzyme activity expressed as 10^{-4} μl O_2 per hr and 10^{-8} g dry weight The enzyme activity per dry weight of the neurons was calculated from the data given in table I and mass data from Brattgård et al (1957)

Days after nerve crush	Operated side	Unoperated side
A Neurons		
Controls	1 4	1 4
6	4 2	5 0
14	6 1	6 4
28	4 3	6 1
48	2 9	4 6
B Glia		
Controls	1 1±0 3	1 1±0 3
	n−8	n=8
6	3 4±0 9*	1 9±0 2
	n 6	n=6
14	2 1±0 3*	2 7±0 8*
	n 10	n−5
28	2 0±0 3*	2 0±0 4
	n 10	n−6
48	2 7±0 6*	1 1±0 4
	n 7	n=5

* $P<0.05$ (from controls i e unoperated animals)

10^{-4} μl O_2 per hr per 2 nerve cells an 1 1 × 10^{-4} μl per 10^{-8} g per hr for the glial samples Since the glial data could be related only to direct dry weight measurements, the succinoxidase activity of the nerve cells was calculated per unit dry weight on basis of the values given by Brattgård *et al* (1957) for hypoglossal neurons (Fig 2)

Effect of nerve crush on the hypoglossal nucleus on the unoperated side

Both nerve cells and glial cells on the unoperated side showed an increase in succinoxidase activity The neurons developed an increased activity up to the 14th day, at which time the succinoxidase activity per nerve cell was about 350 per cent higher ($P < 0.02$) than before operation (Table I Fig 1) The activity expressed per unit dry weight increased conspicuously up to the 14th day (Table II Fig 2) After the 28th day a decrease in succinoxidase activity was seen per nerve cell and per dry weight The activity of the succinate oxidase system in glial samples showed changes with time similar to the changes in neuronal enzyme activity when expressed per dry weight (Table II, Fig 3 The glial increase was however, considerably smaller

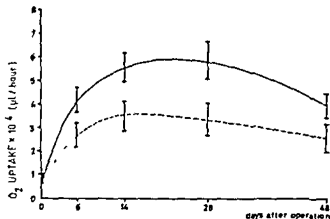


Fig 1 Succinoxidase activity of hypoglossal neurons—correlation between enzyme activity and postoperative time Enzyme activity expressed as 10^{-4} µl O_2 per hr for 2 nerve cells Solid line, mean values for operated side, and interrupted line, mean values for unoperated side Vertical bars indicate standard error of the mean

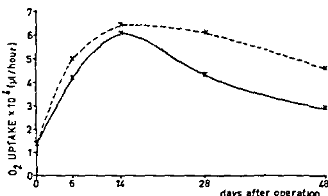


Fig 2 Succinoxidase activity of hypoglossal neurons—correlation between enzyme activity and postoperative time Enzyme activity expressed as 10^{-4} µl O_2 per hr and 10^{-9} g dry weight. The enzyme activity per dry weight of the neurons was calculated from the data given in table I and mass data from Brattgård et al (1957) Solid line mean values for operated side, and interrupted line, mean values for unoperated side

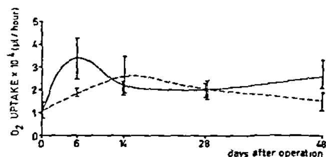


Fig 3 Succinoxidase activity of glial cells from hypoglossal nucleus—correlation between enzyme activity and postoperative time Enzyme activity expressed as $O_2 \times 10^{-4}$ µl per hr per 10^{-9} g dry weight Solid line mean values for operated side, and interrupted line mean values for unoperated side Vertical bars indicate standard error of the mean

Effect of nerve crush on the hypoglossal nucleus on the operated side

After nerve crush a progressive increase was seen in neuronal succinoxidase activity during the first two weeks followed by a more stabilized level of activity During the third and fourth week the activity was about 600 per cent higher ($P < 0.001$) than in control material and about 60 per cent higher than on the unoperated side (Fig 1 and Table I) The 48th postoperative day gave data suggesting a return towards control values When the succinoxidase activity of the nerve cells is calculated per

dry weight the curve expressing the changes in activity with time has a marked peak with a maximum on the 14th day and a slope from the 14th to the 48th day (Fig 2)

The glial samples from the operated side showed increase in the succinoxidase activity compared to material from control animals ($P < 0.05$) during the whole observation period (Table II, Fig 3)

Histochemical observations of enzyme activity in the hypoglossal nucleus after nerve crush

Succinate tetrazolium reductase In the hypoglossal nuclei of control animals and on the unoperated side of experimental animals the activity of succinate tetrazolium reductase was low in comparison with other enzymes tested The staining intensity was slightly higher in the nerve cells than in the surrounding glia (Fig 4 a)

On the sixth day after nerve crush a shift could be seen in the cytoplasmic activity of some regenerating neurons towards an increase in staining intensity in the central parts of the cells These changes were most pronounced on the 14th day but since the differences in distribution of activity were large within the nerve cells the overall change in staining intensity was difficult to assess (Fig 4 b) The general finding, however, was that there were no clear changes in average staining intensity of the neurons on the operated compared to the unoperated side on the 14th day On and after the 28th day the distribution of activity returned to normal with unchanged or slightly decreased staining intensity of the regenerating neurons In the glial cells no clear changes were seen although there was a tendency towards slightly decreased staining intensity in the glial cells compared to the unoperated side after the 14th day

In slices cut from fresh tissue and incubated directly in the medium for succinate tetrazolium reductase a definite increase in activity of the regenerating neurons could be seen on the 14th day

Cytochrome oxidase The cytochrome oxidase activity was similar to that of succinate tetrazolium reductase in being localized in the form of small granules throughout the neuronal cytoplasm The reaction intensity was lower in the nerve cells than in the surrounding glial cells of the hypoglossal nuclei of control animals and on the unoperated side

After nerve crush the cytochrome oxidase activity showed changes comparable to those obtained with succinate tetrazolium reductase

$NADH_2$ tetrazolium reductase (DPN diaphorase) In the hypoglossal nuclei of control animals and on the unoperated side the staining intensity of $NADH_2$ tetrazolium reductase was much higher in the neurons than in the surrounding glial cells The activity was both localized as granules and also observed as a diffuse deposit of diformazan (Fig 5 a) Cold formalin fixation in Hanks balanced salt solution for shorter period of time gave a slight reduction of the reaction intensity but the distribution of activity was the same as in fresh frozen sections

After nerve crush the $NADH_2$ tetrazolium reductase activity showed a progressive increase in the regenerating neurons and their dendrites up to the ninth to 14th day (Fig 6 5) Thereafter during the rest of the observation period the reaction

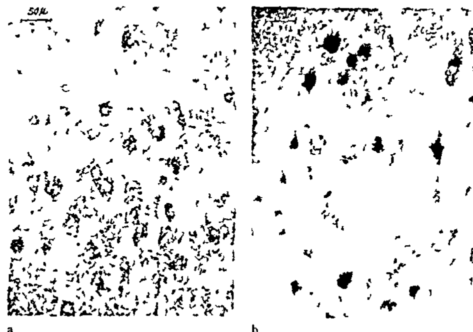


Fig 4 Succinate tetrazolium reductase activity Nitro-BT fresh frozen section 14 days after nerve crush Hypoglossal nucleus a) unoperated side b) operated side

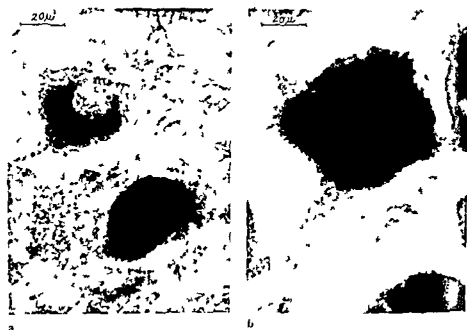


Fig 5. $NADH_2$-tetrazolium reductase activity Nitro BT fresh frozen section 14 days after nerve crush Hypoglossal nucleus a) unoperated side b) operated side

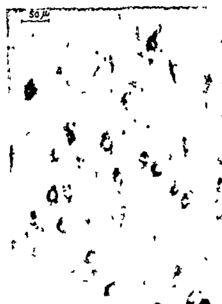


Fig 6 a

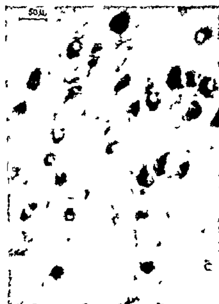


Fig 6 b

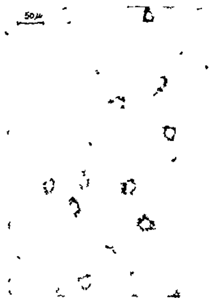


Fig 7 a



Fig 7 b

Fig 6—7 NADH$_2$ tetrazolium reductase activity Nitro-BT fresh frozen sections Hypoglossal nucleus *Fig 6 6 days after nerve crush*
a) unoperated side b) operated side *Fig 7* 48 days after nerve crush a unoperated side, b) operated side

TABLE III. Succinoxidase activity in homogenates of the hypoglossal nuclei on the 14th day after nerve crush. Enzyme activity expressed as μl O_2 per mg wet weight per hr.

Exp. no.	Operated side	Unoperated side
1	1.4	1.3
2	2.0	2.0
3	1.7	1.6
4	1.9	2.0
Mean value ± S.E.M.	1.8±0.1	1.7±0.2

intensity of the neurons remained high (Fig. 7). During this period of increased enzyme activity in the nerve cell bodies an increase in staining intensity was seen also in the axons belonging to the regenerating neurons.

No definite changes could be seen in the glial activity of $NADH_2$ tetrazolium reductase after nerve crush.

In fresh tissue slices cut on the 14th day of regeneration and incubated directly in the $NADH_2$ tetrazolium reductase medium, a conspicious increase of activity was seen in the regenerating neurons.

Succinoxidase activity in homogenates from hypoglossal nuclei

Analyses were only performed on the 14th day. As seen in Table III no significant difference was observed between the operated and unoperated side.

Discussion

The present results on a respiratory enzyme obtained on homogenates of the hypoglossal nucleus do not demonstrate any differences between the operated and unoperated side at a time when the changes of the same enzyme in the neurons are maximal. This finding stresses the importance of analyzing the nerve and glial cells separately.

In a series of works Howe and Mellors (1945), Howe and Flexner (1947) studied the effect of section of the peripheral nerves on the corresponding motor nerve cells. The authors measured succinic dehydrogenase and cytochrome oxidase activity on homogenates of the anterior grey columns from the operated and control sides using the Warburg technique. They found a 10 per cent lower succinic dehydrogenase activity on the operated side 5—6 weeks postoperatively.

It is possible to analyze the supposed discrepancies between results obtained from homogenates and results on single cells, when the large number of variables are considered together. Measurements have shown that the nerve cell bodies on the operated side occupy about 10 per cent of the total volume of the hypoglossal nucleus and on the unoperated side about 6—7 per cent (Sjöstrand, unpublished observations). The enzyme activity per unit dry weight is about three times higher in the nerve cells than in the glial cells. Thus the glial cells contribute the larger part of the en-

zyme activity of the homogenate, although the nerve cells play a role more important than the volume ratio would suggest The microchemical determinations demonstrate that the neuronal and glial succinoxidase activity per weight is very similar on both sides on the 14th postoperative day (Fig 2 and 3) Thus if data related per dry weight (micro-diver) can be directly compared with those related per wet weight (Warburg), the single cell and homogenate results are in accordance The decrease in succinic dehydrogenase found by Howe and Flexner (1947) can possibly corre spond to the decreasing enzyme activity per unit dry weight in the neurons on the operated side with a fairly equal glial cell activity (28th day) in the present study

The unilateral retrograde reaction has over the years been used in several works as an experimental system where the unoperated side has served as a control The concomitant increase in succinoxidase activity of the neurons both on the operated and unoperated side in the present study illustrates the technical limitations when comparisons are made only within the same animal i e in histochemical and radio autographical studies Brattgård and Daneholt (personal communication) found effects on the RNA base ratio in the hypoglossal neurons on the unoperated side following hypoglossal nerve section Electrophysiological data (Green and Negishi 1963) exclude the possibility of cross connections of the axons in the hypoglossal nucleus

The increase in succinoxidase activity per regenerating nerve cell is marked during the third and fourth weeks with an increase of about 600 per cent compared to control material The maximal increase in protein and RNA content per nerve cell which is found during the third to 7th week, is 100 to 150 per cent higher than in control material (Brattgard *et al* 1957) The present results demonstrate that the hypoglossal nerve cell bodies increase their succinoxidase activity 6 times when the RNA and protein content is doubled

The influence of changes in volume and dry weight for evaluation of results related to these parameters is demonstrated in this study The neurons on the unoperated side keep an unaltered level with respect to volume and dry weight (Brattgård *et al* 1957), thus an observed increase in enzyme activity per cells corresponds to the same increase in activity per volume or dry weight On the operated side however the volume and total mass of the neurons change in the same direction as the suc cinoxidase activity The change in enzyme activity expressed per unit volume or dry weight will therefore diminish in proportion to this increase On the 14th postopera tive day the nerve cells on both sides have about the same enzyme activity expressed per dry weight, although the succinoxidase activity per nerve cell on the operated side is about twice as high

The succinate tetrazolium reductase and cytochrome oxidase show a shift in activity distribution between the 6th and 14th day with increased staining intensity in the central region of the nerve cells on the operated side This finding is in accord ance to the perinuclear accumulation of mitochondria shown in micrographs of other regenerating nerve cells Andres 1961 Cervos Navarro 1962 Harkonen 1964) since the precipitates resulting from succinate tetrazolium reductase activity have been

demonstrated in the mitochondria (Sedar and Rosa 1961) The number of mitochondria per unit area has been reported to increase in regenerating neurons when compared to neurons on the unoperated side (Hudson *et al* 1961) Other electron microscopical workers (Cervós Navarro 1962, Harkonen 1964), however, have been unable to show significant changes in the mitochondrial number during regeneration

The mean staining intensity of succinate tetrazolium reductase and cytochrome oxidase do not differ appreciably in the nerve cells on the operated side compared to the unoperated side If the values obtained with the micro-diver technique are calculated per unit volume from data given by Brattgård *et al* (1957) the results obtained for the operated and unoperated sides with the succinate tetrazolium and micro-diver techniques seem to be in accordance The conflicting results obtained for regenerating neurons with the succinate tetrazolium method (Friede 1959, Klein 1960, Fisher and Malik 1964, Soderholm 1965) may be explained by differences in enzyme activity changes, nerve cell volume changes and nerve cell survival in different neuronal groups

The $NADH_2$ tetrazolium reductase activity is markedly increased in the neurons on the operated side (Figs 5—7) with no observable changes on the unoperated side Similar reports are given by Kreutzberg (1963) and Harkonen (1964) The distribution of activity within the cell is generally unchanged for this enzyme The discrepancies between the activity of succinoxidase and succinate tetrazolium reductase on one hand $NADH_2$ tetrazolium reductase on the other may be explained by the location of $NADH_2$-tetrazolium reductase activity both to mitochondria and extramitochondrial structures (Novikoff 1960)

The concomitant increase of $NADH_2$ tetrazolium reductase in the regenerating nerve cell bodies and axons may be interpreted as an intensified transport of material down into the axons In agreement with this interpretation, electron microscopical studies (Takano 1964) have shown activation of the cytomembranes and increased production of neurofilaments in the nerve cell bodies coinciding with the appearance of granular endoplasmic reticulum in the axons of the regenerating hypoglossal neurons during the second week after nerve section in mice

The glial cells react with respect to succinoxidase activity on both sides but the changes are smaller than for the neurons On the operated side a peak of increased glial enzyme activity occurs on the 6th day, i e at a time when the astrocytes and microglia cells are in a phase of hypertrophy and proliferation (Sjostrand 1965 a 1966) Electron micrographs of the satellite cells of regenerating spinal ganglion cells revealed an increase in mitochondrial number and size during this period (Cervos Navarro 1962) Andres (1961) demonstrated similar findings and the frequent occurrence of cytoplasmic indentations, 0 2—0 5 μ long, from the satellite cell into the nerve cell Takano (1964) showed an increase in granular endoplasmic reticulum, vesicles and cell processes of perineuronal oligodendroglial cells during the retrograde reaction after section of the hypoglossal nerve in mice These data indicate an activation of the glial cells during the initial period of nerve regeneration, i e when the

increase in neuronal succinoxidase activity is most rapid This result — in connection with the morphological findings described above — may be interpreted as a closer association between neurons and glial cells

In spite of the extensive glial reaction in the hypoglossal nucleus on the operated side, including astrocyte hypertrophy and reactive microglial changes (Sjostrand 1966) no definite changes in glial enzyme activity can be seen with the histochemical methods used This is in contrast to what is shown for hypertrophic astrocytes and reactive microglial cells in white matter after different types of damage to the brain associated with oedema These cells develop prominent increases in formazan deposits for a variety of respiratory enzymes (Friede 1962, Rubinstein *et al* 1962, Mossakowski 1963) The equivocal results may be due to fundamental differences in cellular reactions in grey and white matter

The present ... Medical Research Council (n o
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Effect of Prostaglandin E_1 on Blood Pressure and Heart Rate in the Dog

Prostaglandin and related factors 48

by

LARS A CARLSON AND LARS ORO

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Abstract

CARLSON, L A and L. ORO *Effect of prostaglandin E_1 on blood pressure and heart rate in the dog* Acta physiol scand 1966 *67* 89—99

Prostaglandin E_1 (PGE_1) was infused into anesthetized dogs in aorta or i v in doses from 0 12 to 1 2 μg/kg/min In all dogs a reproducible fall in blood pressure and increase in heart rate was obtained

ing i v infusion There was no increase in heart rate during infusion of PGE_1 into the animals treated with Agentit[R] or reserpine In dogs receiving continuous infusions of PGE_1 in doses lowering the blood pressure, infusions of noradrenaline or adrenaline still induced a marked rise in blood pressure Infusion of the sympathetic ganglionic stimulating agent DMPP or electrical stimulation of the central ends of the cut vagus nerves also raised the blood pressure in dogs receiving PGE_1 The hypothesis was discussed that PGE_1 lowers the blood pressure by actions independent of catecholamines

Goldblatt (1933, 1935) and Euler (1934, 1935 1936) independently demonstrated the presence of a vasodepressor and smooth muscle stimulating activity in extracts from seminal fluid and from male vesicular glands The name prostaglandin was attributed to the active principle by Euler (1935) who also showed that it was fat soluble and had acid properties

The chemical work on the factor prostaglandin has been continued during recent years by Bergstrom and his group and the prostaglandins have been isolated and their chemical structure elucidated (*cf* Bergstrom and Samuelsson 1965) It has been found that the factor prostaglandin consists of several chemically related compounds, called prostanoic acids which are present in various male and female organs

TABLE I Mean blood pressure (mm Hg) and heart rate (beats/min) during infusions of PGE_1 In high in aorta and the second low in aorta and in C (5 dogs) the first infusion was given 45 min between the two infusions The dose of PGE_1 was the same in both infusions but

		Experiment A			
		High in aorta First infusion		High in aorta Second infusion	
Route of administration		Before	Change*	Before	Change*
Blood pressure	M±SEM	153±8	-17± 5	148±6	-16± 5
	Range	130-180	-35--5	130-170	-40--5
Heart rate	M±SEM	147±9	40±10	139±7	44±9
	Range	122-182	22-86	120-162	24-76

* = Calculated on the individual changes from the level immediately before to the level 5 min after
M = Mean value, SEM = Standard error of the mean

Euler (1934, 1936, 1939) showed that the factor prostaglandin lowered the blood pressure and induced a vasodilatation in the perfused hind leg of cat Infusion of prostaglandin E at a rate of 0 2—0 7 µg/kg/min to two healthy subjects induced a blood pressure fall and increase in heart rate (Bergström *et al* 1959 a), while infusion of PGE_1 at rates of 0 1—0 2 µg/kg/min to man only induced an increase in heart rate (Bergström *et al* 1965 a, b)

Euler (1939) also reported that the factor prostaglandin reduced the blood pressure rise induced by a single injection of adrenaline in the cat Simultaneous administration of PGE_1 and adrenaline to dogs was found to prevent the adrenaline induced rise in blood pressure (Bergstrom, Carlson and Oro 1964). It was also shown that PGE_1, PGE_2 and PGE_3, but not $PGF_1\alpha$, lowered the blood pressure during continuous infusion of noradrenaline into anesthetized dogs (Bergstrom *et al* 1964)

The question arose if the PGE_1-induced blood pressure fall observed under various conditions was due to an effect independent of catecholamines or due to an inhibition of the actions of catecholamines on the blood vessels In the present work the role of catecholamines and the sympathetic nervous system for the effects of PGE_1 on blood pressure and heart rate was studied by infusing PGE_1 to anesthetized dogs, untreated or pretreated with sympathetic blocking agents and also by following the effect of PGE_1 on changes in blood pressure induced by catecholamines A preliminary report on the results has been given (Carlson 1966)

Methods

exp A (6 dogs) two infusions were given high in aorta in B (5 dogs) the first infusion was given high in aorta and the second in a common carotid artery PGE_1 was given for 5 min with at least varied between the dogs from 0 2—1 0 $\mu g/kg/min$

Experiment B				Experiment C			
High in aorta		Low in aorta		High in aorta		In a common carotid artery	
Before	Change*	Before	Change*	Before	Change	Before	Change*
151±8	−27±4	145±8	−11±4	145±7	−42±8	140±8	6±4
130−180	−40− 20	125−170	−25−0	120−160	−70− −25	125−160	−5−15
159±2	31±9	148±4	16±4	148±8	34±5	152±10	12±4
152−165	4−56	136−160	8−26	116−164	20−46	116−174	4−26

the start of the infusions

Adrenaline and *isoprenaline*-sulphate were used in the form of commercial preparations from ACO (Sweden), 1 mg/ml

DMPP, dimethylphenylpiperazinium bromide was received from Fluka (Switzerland)

Reserpine was received from Ciba (Sweden) as Serpasil[R], 2 5 mg/ml Hexamethylene hydroxyethyl dimethylammon chloride a sympathetic ganglionic blocking agent was received as *Agentil*[R] from Recip (Sweden)

Propranolol a beta adrenergic blocking agent was received from AB Meda (Sweden) as Inderal[R], 5 mg/ml

The substances were diluted in physiological saline before administration

Procedure

Adult mongrel dog
for at least 18 hrs T
b w with suppleme
induce anesthesia
in the brach al or femoral artery for connection with an Elema Schonander pressure transducer (EMT 490 A) for blood pressure registration The mean blood pressure has been calculated as the mean value of the systolic and diastolic pressure One or two further catheters were placed into superficial veins for administration of the various substances When infusions in aorta were done a catheter was put into a femoral artery via a small arterial branch The infus ons h gh in aorta were given with the catheter tip placed in the thoracic aorta at approximately the same level as the heart The catheter tip was d splaced 15—25 cm downwards when PGE_1 was given low in aorta For administration of PGE_1 into the carotid artery a teflon catheter was introduced into the left common carotid artery at the level of the thyroid gland by the Seldinger technique (1953 The tip of the catheter was about 5—10 cm below the carotid sinus and sal ne was infused into the artery before and after the admin istrat on of PGE_1 at the same rate as PGE_1 solution

Results

Effects of infusion of PGE_1 in the aorta, intra enously and in a carotid artery

Administration of PGE_1 in aorta, in doses from 0 12 to 1 2 $\mu g/kg/min$ lowered the arterial blood pressure and increased the heart rate in all dogs The administration

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Acta physiol. scand. 1965. 64. 87—92

From the Institute of Medical Physiology University of Copenhagen, Denmark

The Magnitude of the Skimming Phenomenon in the Interlobular Arteries of the Cat Kidney

By

Ole I. Nissen

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Abstract

Nissen, O I *The magnitude of the skimming phenomenon in the interlobular arteries of the cat kidney* Acta physiol scand 1965 *64* 87—92 — Samples simultaneously collected from the arterial system, from the deep renal veins and from the subcapsular renal veins in the cat kidney have been analysed with respect to red cell volume, plasma proteins, plasma osmolality (and plasma PAH) The ratio subcapsular venous concentration/arterial concentration was on an average 1 10 for the red cells, 1 09 for the plasma proteins and 0 99 for plasma osmolality The ratio deep venous concentration/arterial concentration was on an average 0 90 for the red cells, 0 89 for the proteins and 1 03 for the osmolality The difference between the protein ratios and a [illegible]

be explained from the alterations in the tonicity of the blood during its passage through the kidney

In 1956 Pappenheimer and Kinter proposed their wellknown cell separation theory based on the phenomenon of plasma skimming described by Krogh (1929, p 6) This skimming should occur in the interlobular arteries, the outer glomeruli then receiving a cell rich component of the blood and the deeper glomeruli a cell poor component.

A direct consequence of this cell-separation theory is that the blood leaving the superficial cortex should be cell-rich and that the blood flowing from the inner part of the cortex (and the medulla), cell-poor

According to v Möllendorff (1930, pp 133—135) the venous drainage of the cat kidney is characterized by the fact that the blood from a considerable, outer, part of the cortex passes to the well developed subcapsular veins which, confluenting, lead it along the surface of the kidney to the renal vein. The venous drainage from the medulla and the inner part of the cortex takes place, as in other mammals, through the arcuate veins

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analyses not reported here the hematocrit determinations were omitted. Donor blood was

correction for trapped plasma was made The standard deviation determined from duplicates

The osmolality of the plasma was determined by the method of freezing point depression (Advanced Instruments Inc) The standard deviation determined from duplicates was 0.2 per cent of the mean (N = 33

In most of the experiments urine was collected via a bladder catheter The left ureter was ligated. The urine flow rates were purposely kept low, usually they amounted to about 0 1 ml per minute, consequently the loss of fluid by urine formation can have had only a negligible effect on the hematocrit and plasma protein concentration of the renal venous blood.

After the experiment the position of the deep venous catheter tip was determined. In all the experiments it was lying well (more than 0.5 cm) distal to the outlets of the subcapsular veins in the renal vein.

Results

The data presented derive from 11 expts Protein concentrations as well as hematocrits were determined in 19 periods in 7 expts. (cf p 3 line 5—6) The results are shown in Fig 1 The ratio between the protein concentrations of the deep or the superficial venous plasma and that of the arterial plasma (the "fractional concentration") is plotted as the abscissa, while the ordinate represents the corresponding ratio for the hematocrit values The figure contains 2 more points for superficial than for deep blood, because valve formation at the catheter tip made collection of deep blood impossible in one of the 7 expts

The average value for the fractional protein concentration in plasma from the subcapsular venous system was 1 09 and the average fractional hematocrit value in this blood was 1 10 The corresponding figures for the deep venous blood were 0 89 and 0.90

The long dashed curve represents the relationship between the ratios to be expected if the only factor causing deviations of the ratios from one were a net removal of a protein- and erythrocyte free isotonic fluid from the arterial blood or a net gain of such fluid. The average hematocrit value 34 per cent range 23—44 was used for calculation of the curve The slope of the curve is less than 45 because such fluid loss or gain would affect only the plasma volume and because the hematocrit is a concentration per unit volume of total blood whereas plasma protein concentration is given per unit volume of plasma, for the same reasons the curve is curvilinear

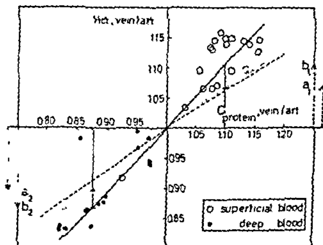


Fig 1 *Abscissa* o concentration of protein in subcapsular venous plasma/concentration of protein in arterial plasma ● concentration of protein in deep venous plasma/concentration of protein in arterial plasma

Ordinate o hematocrit in subcapsular venous blood/hematocrit in arterial blood ● hematocrit in deep venous blood/hematocrit in arterial blood

For details confer the text

The fractional osmolality of the plasma samples (venous/arterial) was determined in 7 periods In the superficial plasma this figure was on an average 0 99 (range 0 96—1 00), in the deep plasma 1 03 (range 1 00—1 06) The difference between the two types of venous plasma may in all essentials be accounted for by differences in the concentrations of sodium ions with matching anions (unpublished data) As the erythrocyte membrane is virtually impermeable for sodium ions, a hypotonicity in the plasma due to a lowering of the concentrations of these ions will cause a swelling of the erythrocytes

Glomerular filtration as such will cause a movement of the hematocrit-protein concentration relationship from point 1 0—1 0 in Fig 1 along the dashed curve towards the right Subsequent regain of an isotonic fluid will lead to a backward displacement along the same curve towards or past the 1 0—1 0 point

However, the fact that the venous blood is non isotonic indicates that the fluid, which the blood — upon filtration — has regained by reabsorption, has been non-isotonic viz hypotonic in the case of the superficial blood and hypertonic in the case of the deep blood

Consequently in the former case the covariation of the hematocrit ratio and the protein concentration ratio will be described by a curve with a smaller slope than the dashed curve, since a swelling of the red cells takes place A correction for this fact — based upon the average fractional osmolality — has been applied in Fig 1 (upper dotted curve) Similarly a correction for the hypertonicity of the deep blood has been applied (lower dotted curve)

The full-drawn line represents the relationship between the ratios, estimated from the experimental data The ratio $a_1 + a_2/b_1 + b_2$ gives a rough measure for that part of the difference between the hematocrit ratios which can be accounted for by processes other than skimming

In 11 periods in 7 expts the arterial plasma concentrations of PAH were below 5 mg per cent The average extraction fraction (defined as 1 — concentration of PAH in venous plasma/concentration of PAH in arterial plasma) for PAH in the superficial venous plasma at these concentrations was 0 94 ± 0 02 (S D, n = 11), the corresponding value for the deep venous plasma was 0 82 ± 0 04 (S D, n = 11) In one additional experiment the plasma concentration was below 5 mg per cent (namely 2 1 mg per cent) but in this the extraction fractions (0 73 for the superficial plasma and 0 55 for the deep plasma) deviated so much from the others that these values were omitted in the calculations of the averages and standard deviations

The average blood pressure was 125 mm Hg (range 92—152)

Discussion

From the difference between the concentrations of plasma proteins, plasma PAH and red corpuscles in the superficial and deep venous blood it is concluded that the areas drained by the two venous systems differ considerably with respect to function

The very high extraction fraction of PAH (94 per cent) in the plasma drained from the peripheral part of the cortex indicates that at most 6 per cent of this plasma may pass through nonextracting tissues or "Pappenheimer shunts" Further discussion of the PAH extraction is postponed to a subsequent paper

According to the cell separation theory of Pappenheimer and Kinter (1956) the hematocrit of the blood leaving the peripheral parts of the cortex should be considerably higher than that of arterial blood A single sample of blood from a subcapsular vein of the cat kidney was examined by these authors (p 383 in their paper), they reported the hematocrit to be 'normal', but did not discuss the implications for the theory

Ulfendahl (1962) found the hematocrit values of subcapsular venous blood to be some 10 per cent higher than those of arterial blood He considered this as due to plasma skimming, however, he pointed out that the percentage difference between the hematocrit values was much too small to fit into the cell separation theory of Pappenheimer and Kinter Samples form the deep renal venous system were not investigated

The present experiments confirm that the hematocrit value of the blood drained from the peripheral cortex is higher than that of arterial blood, and add complementary information on the hematocrit of deep venous blood

However a different conclusion has been arrived at from the present experiments because factors, other than skimming, that may affect the venous hematocrit values has been taken into consideration

These are the following

1) The blood plasma traversing the outer part of the cortex may loose more fluid by glomerular ultrafiltration than it gains by reabsorption and the opposite may hold for the blood plasma passing through the deep parts of the kidney This would *alter the hematocrit and the plasma protein concentration in the same direction* and the alterations would be correlated as indicated by the long dashed curve in Fig 1
2) If the blood traversing the outer or the inner part of the kidney regains — by reabsorption — a fluid which is hypotonic or hypertonic (relative to arterial blood), a movement of water between the red cells and the plasma would take place The

occurrence of this phenomenon in the kidney is indicated by the fact that the osmolalities of the deep and superficial venous blood differ significantly from that of arterial blood By further taking this factor into consideration (using the average osmolalities of superficial and deep venous blood) the fractional hematocrits and the fractional protein concentrations should be correlated as indicated by the two short, dotted curves in Fig 1 — provided no skimming occurred

(The author has disregarded the formation of renal lymph as a factor causing any significant changes in the hematocrit and protein concentration of the renal venous blood, because of the smallness of the lymph flow in comparison with the renal blood flow)

By comparing the distances $a_1 - a_2$ and $b_1 - b_2$ in Fig 1 it may be seen that the two above mentioned phenomena account for the major part of the observed rather small deviations of the fractional hematocrits from one From this it must be concluded that plasma skimming in the interlobular arteries as proposed by Pappenheimer and Kinter takes place at most to an negligible extent

The author is greatly indepted to professor Poul Kruhøffer for criticism of the manuscript.

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The Effect of Osmotic Pressure Changes on the Isolated Muscle Spindle

By

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Abstract

Ottoson, D *The effect of osmotic pressure changes on the isolated muscle spindle* Acta physiol

sensitivity to stretch It is concluded that the observed changes are mainly due to osmotic effects on the sensory endings of the spindle The results suggest that the endings behave as simple osmotic sacs within a limited range of osmotic strengths and that the sensory membrane is equally sensitive to distention and folding

The muscle spindle responds with a burst of impulses when stretched or when the intrafusal fibres contract It is generally accepted that this response arises as a result of the deformation of the endings of the afferent fibre At present but little is known about the kinetics of the mechanical events underlying the excitatory process There seems to be reason to believe that the conversion of the mechanical stimulus into the graded electrical response (Katz 1950 a, b) occurs in the membrane of the bulbous expansions of the ramifications of the nerve fibre (Katz 1961) Since the end bulbs vary in size as well as in their structural relation to the intrafusal fibres, it seems most unlikely that all endings are affected to the same extent by the elongation or shortening of the muscle fibres The sustained potential recorded from the nerve during stretch can therefore be presumed to represent the total integrated effect of a differential distortion of various

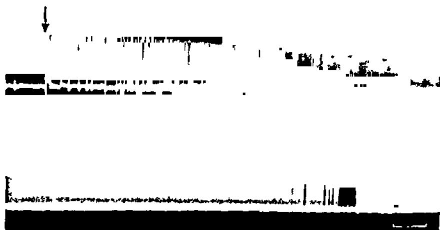


Fig 1 Typical effect of strong hypertonic solution Continuous record on moving film At arrow one small drop of a 5 × R sucrose solution was added to the bath Time calibration 1 sec

parts of the sensory membrane In view of the structural organization of the spindle it may also be assumed that the external stimulus undergoes a transformation before the sensory endings are reached The parameters of the external stimulus consequently represent an inaccurate measure of the characteristics of the actual stimulus

It was thought that by changing the osmotic pressure of the external solution it would perhaps be possible to obtain a uniform and also a quantitatively more well defined stimulus than by stretch As will be reported in the present paper it was found that the spindle could be excited by osmotic pressure changes and also that charac teristic alterations of the sensitivity to stretch were produced by anisotonic solutions A preliminary report of the results has been published earlier (Ottoson 1964)

Methods

Preparation The experiments to be described were carried out on isolated spindles of the

l fied over into a small chamber (0 5 × 2 mm) where it was clamped at each end to a thin plastic rod One of the rods was connected to a micromanipulator so that the length of the muscle could be changed

In order to prevent contractions or changes in the tonus of the intrafusal fibres the muscle bundle was crushed close to the ends of the spindle Sometimes the fibres also had to be punc tured by a glass capillary so as to eliminate fibrillations Stretch of 250 msec or 3 sec durat on was applied by means of an electromagnet connected to the spindle by one of the plastic rods Before exposure to the test solution the response to stretch was recorded at regular intervals and no test were made until the response in Ringer s solution had remained constant for at least 10 min

Recordings The impulses of the afferent nerve were recorded with silver chloride agar electrodes One electrode was placed in the fluid in the chamber while the other was appl ed to the sensory axon which was lifted up in oil The electrodes were connected to a Grass P 6 ampl fier and a Tektronix 502 oscilloscope DC record ng was used in all experiments with test stretches

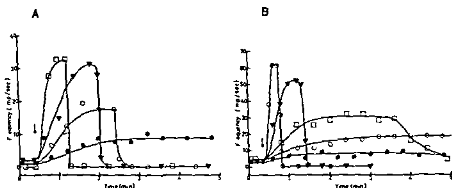


Fig 2.1 Effects of hypertonic sucrose solutions of different concentrations. Ordinate average frequency of discharge Change from iso- to hypertonic fluid marked by arrow Concentrations expressed relative to Ringer's fluid ●—● = 1 5 × R, ○—○ = 2 × R, ▼—▼ = 3 × R, □—□ = 5 × R
B Effect of hypertonic NaCl Ringer's solution Concentrations ●—● = 1.25 × R, ○—○ = 1 5 × R, □—□ = 2 × R, ▼—▼ = 3 × R, ◑—◑ = 6 × R

up by replacing sodium with choline chloride on a mole for mole basis All solutions were made up fresh before use Hypotonic solutions were made by adding distilled water to Ringer's

solution To obtain a complete replacement of the Ringer's fluid several changes had to be made This could usually not be carried out in less than half a minute It was therefore not

known All experiments were carried out at room temperature

Results

Hypertonic solutions

Effects on the resting spindle The change from Ringer's solution to a hypertonic solution regularly elicited an increased activity of the spindle (Fig 1) This effect, in the following called the hypertonic discharge, was usually not obtained until the osmotic pressure was raised to 1 5 × R In some preparations, however, an increase to 1 25 × R was sufficient to increase the firing rate of the spindle The effect appeared within 10 to 30 sec after the change to the hypertonic solution With 1 5 R the discharge increased slowly to 10 to 15/sec and in most cases remained constant at this frequency for several minutes With further increase of concentration the response appeared with shorter latency, the rise became faster and the peak frequency higher (Fig 2) Typically the

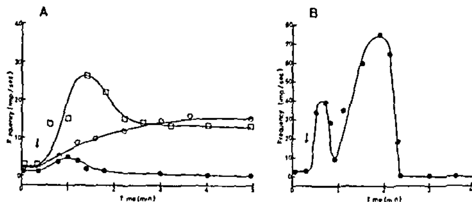


Fig 3 ...
one pr ...
(□—□ ...
solution ...
osmolarity (2 × R) and applied in the order ○—○, □—□, ●—● A final control test with sucrose Ringer's solution gave a response identical to that shown by the curve ○—○.
B Double discharge evoked by a *hypertonic* (6 × R) *NaCl-Ringer's solution*

duration of the discharge also became successively shorter as the osmotic pressure was increased With concentrations above 2 × R the firing generally stopped abruptly and the spindle then remained silent as long as it was immersed in the hypertonic solution The maximum response in terms of peak frequency was most often obtained with 3 to 4 × R but *sometimes not until the concentration was raised to* 6 × R *The increased* activity of the spindle was usually associated with an increased frequency of miniature potentials (Katz 1950 a)

In order to ascertain that the observed effects were not due to contractions of the intrafusal bundle, the preparation was observed under the microscope in a number of *experiments during and after the change from isotonic to hypertonic solution in the* bath In no case was it possible to detect any contractions or movements of the fibres which could explain the excitatory effect The fact that in all experiments the muscle fibres were pinched close to the polar ends of the spindle also reduces the likehood of muscular contractions as the cause of the afferent discharge

Hypertonic NaCl solutions had essentially the same effect as the sucrose solutions (Fig 2B) In general, however the NaCl solutions were active in lower concentrations than the sucrose solutions With NaCl solutions the effect also appeared earlier and the frequency of the discharge rose faster than with sucrose solutions of corresponding osmolarity It was also possible to drive the spindle to higher frequencies with NaCl *solutions than with sucrose solutions When the osmotic pressure was raised by* adding choline chloride to Ringer's fluid the hypertonic effect was similar to that obtained with sucrose

The influence of the Na concentrations of the bathing fluid on the hypertonic effect was examined in a series of experiments in which the spindle was exposed to *hypertonic sucrose solutions with different Na concentrations,* the NaCl being replaced by choline chloride It was found that a lowering of the Na^{+} concentration be-

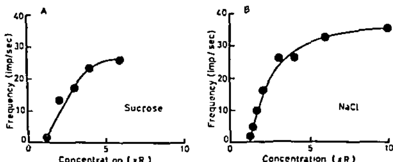


Fig 4 Relation between osmotic pressure and discharge Ordinate peak frequency of discharge Abscissa osmotic pressure in units relative to Ringer's solution Results obtained from two different preparations *A* sucrose Ringer's solution, *B*, NaCl Ringer's solution

low the value of normal Ringer's solution was regularly followed by a diminution and shortening of the hypertonic effect (Fig 3A)

With high concentrations the spindle sometimes responded with two bursts instead of a single discharge (Fig 3B) The initial discharge was usually brief and separated from the second, more prolonged one, by a short interval of lower firing rate In some preparations there was a silent pause in between the two bursts The double response was most often seen with hypertonic NaCl solutions but also appeared with sucrose solutions

Recovery After treatment with low concentrations the spindle recovered completely when returned to Ringer, and repeated exposures were usually followed by only a small decline of the response provided the spindle was left to recover in Ringer's solution for sufficient time after each treatment After exposures to high concentrations (above 2 × R) recovery was less complete and the response to subsequent applications became successively smaller Repeated treatments with solutions above 2 × R were also typically accompanied by a gradual reduction of the duration of the response

To minimize exhaustion of the spindle the solutions to be tested were applied in the order from lower to higher concentrations When strong solutions were used the time of exposure was kept as short as possible After each exposure the spindle was left for at least 20 min in Ringer's fluid and with higher concentrations for 30 to 40 min between the exposures By these precautions the deleterious effects of repeated immersions could be minimized

Relation between osmotic pressure and discharge Since the spindle did not recover completely after immersion in strong solutions it was difficult to establish the relation between the osmotic pressure and the increase of activity of the spindle This difficulty could be partly overcome by prolonging the recovery period in Ringer's fluid and shortening the soaking time Some preparations were also more resistent than others and in these cases it was possible to obtain comparable data for a relatively wide range of concentrations The curves in Fig 4 give examples of the results obtained in two such experiments As seen, the peak frequency of the discharge rises almost linearly with increasing osmotic pressure up to 4 × R The curves also illustrate the typical differences between the effects of sucrose and NaCl

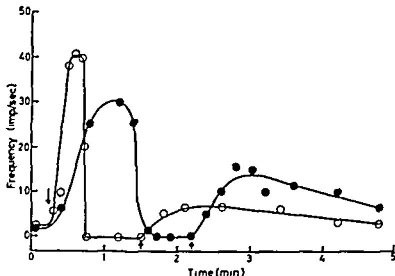


Fig 5 Post-hypertonic effects on return to Ringer's fluid after soaking in NaCl Ringer's solution Change from iso- to hypertonic solution marked by first arrow from left, return to Ringer's fluid by second and third arrow respectively Concentrations relative to Ringer's fluid ●—● = 3 × R, — = 5 × R

Post-hypertonic effects The behaviour of the spindle when soaked in Ringer's solution after exposure to increased osmotic pressure varied with the concentration of the hypertonic solution and with the time of exposure A spindle that had been treated with a weak hypertonic solution for a few minutes usually returned to its original spontaneous firing rate within 10 to 20 sec whereas after prolonged immersion the recovery took place more slowly In many preparations, however, washing with Ringer's solution produced an increased activity (Fig 5) which was sometimes maintained for several minutes This type of response, which varied from one preparation to another, was obtained only after treatment with strong hypertonic solutions and only when the change from the hypertonic solution to Ringer's fluid was made after cessation of the hypertonic discharge In the experiments illustrated in Fig 5, 3 × R gave a stronger 'off' response than 5 × R In other experiments the intensity of the discharge increased with increasing osmotic pressure

Effects on the response to brief stretch Increase of the osmotic pressure of the bathing solution regularly caused a reduction of the response of the spindle to stretching This effect was noticeable at a 50 per cent increase of the osmotic pressure and became gradually more pronounced with stronger solutions (Fig 6) In the initial phase of the action of strong solution when the spindle discharged vigorously stretch usually failed to produce any response (Fig 6, *I*) In the later phase of the hypertonic discharge when the activity had declined stretch regularly caused an inhibition of the maintained discharge Still later, after cessation of the hypertonic discharge, stretching usually elicited one single impulse at the onset of stretch (n)

The time course and magnitude of the change of the response in hypertonic media are illustrated by the curves in Fig 7 The responses are expressed as percentages of

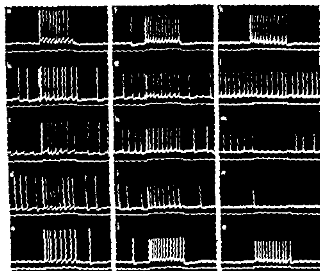
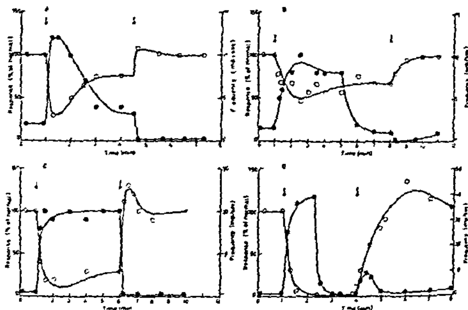


Fig 6 Typical effects of hypertonic solutions of response of muscle spindle to brief (250 msec) stretch *a* response in Ringer's fluid, *b*—*d* after 0 5, 2 and 5 min in NaCl Ringer's solution (1 5 × R), *e* 5 min after return to Ringer's fluid, *f*—*j* similar trials with 2 × R and *k*—*o* with 3 × R solutions Duration of stretch indicated by lower tracing

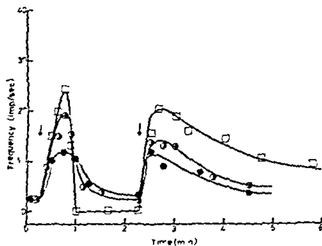
the number of spikes elicited in Ringer's fluid by the test stretch When calculating the effect of the various solutions tested, the average number of spikes of the hypertonic discharge in a period corresponding to that of stretch was subtracted from the total number of spikes in the stretch period In addition to the curve showing the change of the response, the curve of the hypertonic discharge also is given in the figures The curves in A and B give two examples of the reduction of the response in a 1 5 × R solution With solutions 2 to 3 times the normal osmotic pressure the depressive effect became still more pronounced, as illustrated by the curves in C and D As seen there is still a residue of 15 to 20 per cent of the original response in 2 × R, while in 3 × R the block is complete It may also be noted that return to Ringer's fluid is followed by a transient phase of increased responsiveness

Hypotonic solutions

Effects on the resting spindle Immersion in solutions of low osmotic pressure gave rise to a transient increase of the activity of the spindle In most cases this effect was not obtained until the osmotic pressure had been reduced to 50 per cent of that in Ringer's fluid but sometimes a reduction of 25 per cent was sufficient to elicit a response As a rule, the discharge frequencies of the hypotonic responses were lower than those of the hypertonic responses with a 0 5 × R solution the discharge usually did not exceed 15 sec As the osmotic pressure was further reduced the response increased (Fig 8), with 0 1 × R solution firing rates of 20 to 40 sec were obtained With diminishing osmotic pressure the duration of the response also became successively shorter A 0 5 × R solution usually produced an increased activity for 1 to 1 5 min followed by a slow decay, while a 0 1 × R solution usually gave a short burst of impulses after which the spindle remained silent as long as it was kept in the solution The type response described above was obtained in 40 out of the 44 spindles tested with hypotonic solutions In the remaining 4 spindles application of hypotonic solutions produced a decline of the



... (open circles) ...



... average frequency of discharge. Change from ... Ringer's fluid by second ... R, ... = 0.25 × R, ...

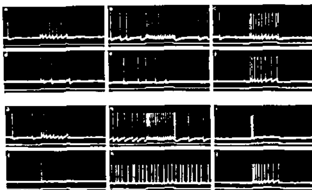


Fig 9 Typical effects of hypotonic solutions on response of muscle spindle to brief (250 msec) stretch, *a* response in Ringer s fluid, *b*—*d* after 0.5 2 and 5 min in 0 5 × R *e* and *f* 0 5 and 5 min after return to Ringer s fluid Records *g*—*l* similar run with 0 25 × R Duration of stretch indicated by lower tracing

activity Since these spindles maintained an unusually high spontaneous firing rate (8 to 12/sec) in Ringer s fluid it seems most likely that they had been damaged during the dissection procedure It is noteworthy that though these spindles did not respond to a reduction of the osmotic pressure they reacted quite normally to hypertonic solutions

Post hypotonic effects The change from hypotonic to isotonic fluid regularly elicited an increased activity of the spindle (Fig 8) The response appeared within a few seconds after return to Ringer s fluid and was maintained for several minutes With successively [illegible]

time of exposure

Effects on the response to brief stretch The records in Fig 9 illustrate the typical effects evoked by soaking the spindle in hypotonic solutions Immersion in a 0 5 × R solution produced the usual increase of activity followed by a final cessation of the discharge As shown by record *b*, the response to stretch is enhanced in the period of hypotonic discharge As the latter decays the response diminishes and after cessation of the hypotonic discharge stretch only produces a few impulses (*d*) Return to Ringer s fluid gave the usual effect *viz* a short lasting increase of activity Stretch applied in this period often failed to produce any noticeable effect on the spindle or elicited only a few impulses (*e*) With the decline of the posthypotonic discharge the response appeared again attaining its original size in about 5 min With further decrease of the osmotic pressure of the external fluid the effects on the spindle became more marked The enhancement of the response in the period of hypotonic disharge (*h*) was followed by a pronounced reduction until only one impulse was left (*j*) Washing in Ringer s fluid elicited an intense post hypotonic discharge which persisted unchanged during stretching (*k*) The time course and magnitude of the effects elicited with hypotonic solutions appear in detail from Fig 10

The curves in Fig 11 summarize the results obtained in the present study Curve A illustrating the excitatory action of solutions of different osmotic strengths gives the mean values from 16 experiments in which the recovery of the spindle after each of the exposures was almost complete thus allowing tests to be carried out within the range from 4 R to 0 25 R As appears from curve A [illegible] side of the

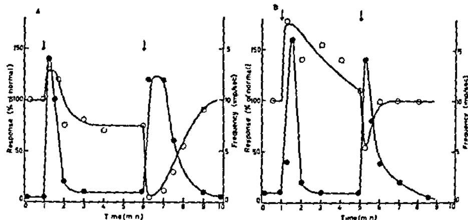


Fig 10 Changes of response to stretch (open circles) in 0 5 × R (*A*) and 0 25 × R (*B*) Filled circles show changes in frequency of maintained discharge Response values expressed as in Fig 9 Hypotonic periods between arrows

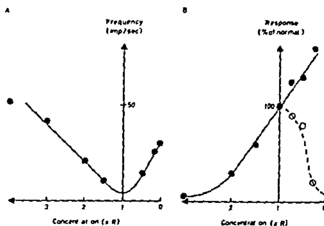


Fig 11 *A* relation between osmotic pressure and discharge of spindle Composite results of 16 experiments (8 with hypertonic and 8 with hypotonic solutions) Ordinate peak frequency of discharge Abcissa osmotic pressure of the bathing fluid *B* responsiveness of muscle spindle at different osmotic pressures Ordinate response expressed in per cent of that obtained in Ringer's fluid as in Fig 9 Abscissa osmotic pressure of bathing fluid in units relative to Ringer's fluid Dotted line shows late decrease of sensitivity in hypotonic solutions

isotonic point lead to an increased activity Though a comparison between the effects of increased and decreased pressure is limited to a small range, the results suggest that osmotic changes of the same order of magnitude in opposite directions have about the same action This indicates that the sensory membrane is equally sensitive to folding and distention Curve B illustrates the responsiveness of the spindle to stretch when kept in solutions of different osmotic pressures As seen the response of

the spindle to a given stretch is linearly related to osmotic strength within the range from 2 × R to 0 25 × R The close correlation between excitatory action and responsiveness on one hand and osmotic pressure on the other indicates that the changes underlying the action of anisotonic media closely follow the laws governing osmotic processes

Discussion

The experiments described above demonstrate that changes in osmolarity have an excitant action on the muscle spindle when the pressure is raised or reduced by more than 25 per cent of that of Ringer s solution As also shown increased osmotic pressure results in a reduced sensitivity to stretch while reduced pressure gives rise to a transient increase followed by a decrease in responsiveness Likewise return back to Ringer s solution after immersion in anisotonic media excite the spindle and produce changes in its responsiveness to stretch The simplest explanation of these effects seems to be that they arise as a result of a mechanical deformation of the sensory endings caused by volume changes of the different tissues forming the spindle The effects may also be produced by contractions of the intrafusal fibres but this explanation is less likely *since the muscle fibres were always pinched before exposing the spindle* to the test solution Also direct observations of the spindle did not in any case disclose any contractions

As shown by Katz (1961) in an electronmicroscopical study of the frog s muscle spindle the terminal branches of the sensory fibre form bulbous expansions which are seated in cup-like depressions of the muscle fibres to which they are attached by thin strands of connective tissue while other bulbs are lying free in the lymphatic space Out of the number of factors which may contribute to the effects observed the volume changes of the sensory end bulbs and the intrafusal fibres are probably the most important Since many of the sensory bulbs are only loosely-attached to the muscle membrane it does not appear to be very likely that the excitatory effects and the changes in sensitivity to stretching are primarily to be ascribed to the volume changes of the muscle fibres The conclusion would consequently be that the changes in activity of the spindle when exposed to anisotonic media are mainly due to osmotic effects on the sensory end bulbs

In addition to the direct osmotic effect on the sensory endings changes in osmotic pressure will also affect the afferent fibre It has been demonstrated (Stampfli 1955 Stampfli and Nishie 1955 Schmidt and Stampfli 1959) that hyperosmotic media produce a reduction of the membrane potential of myelinated fibres whereas hypo-osmotic media have the opposite effect It is of interest to note that the hypotonic effect was also obtained by switching from a hypertonic solution back to isotonic solutions (Schmidt and Stämpfli 1959) It has further been demonstrated by Sato (1950) that a nerve treated with hypertonic media responds to a constant current with a repetitive discharge If it is assumed that the excitant action is caused by the distortion of the sensory membrane it seems most likely that this action is modified by the changes in the membrane potential of the nerve

The changes in sensitivity to stretch may be explained in a similar way When soaking the spindle in a hypertonic medium the sensory endings shrink as water leaves the cells and the membrane becomes folded Stretching a hypertonic spindle can be

assumed to result in the opposite effect, i.e. a distention of the membrane. In the case of a weak hypertonic action stretching would produce a response, while in a strong hypertonic medium stretch would result in an unfolding of the membrane and consequently lead to a reduction of the stimulus as represented by the folding. In this case stretch would be expected to cause an inhibition of the hypertonic discharge. As described above this effect was observed during a transient phase of the hypertonic period when the spindle was bathed in strong hyperosmotic media. The mechanisms underlying the changes in sensitivity to stretch in hypotonic media seem to be analogous to those responsible for the hypertonic effects. However, the events in hypotonic media are no doubt more complex since in this case the osmotic action is influenced by the effect caused by deficiency of ions essential to the function of the spindle. The effect of hypotonic solutions is similar to that caused by stretch insofar as the sensory membrane is distended in both cases. The situation when the hypotonic spindle is being stretched may thus be compared with what happens when a short stretch is superimposed upon a sustained stretch. The increase of the response in the initial phase of the hypotonic period would accordingly correspond to the increased responsiveness of the spindle under maintained stretch.

If the sensory endings behaved as ideal osmometers it would be possible to calculate the volume changes of the sensory endings. It has been demonstrated (Sato 1954, Reuben *et al.* 1963) that there is a linear relation between osmotic strength and relative volume of single muscle fibres in hypo-osmotic media. Recent studies on the effect of hypertonic solutions on single fibres (Dydynska and Wilkie 1963) have further shown that a similar relationship exists up to 2 × R. This correlation is absent in stronger solutions probably because of damage to the fibres. The results obtained in the present study indicate that within a limited range of osmotic strengths the sensory endings behave as simple osmotic sacs. In assessing the effects observed it has however to be taken into account that the changes in volume are not due to passage through the membrane of water alone. Together with the flow of water there is most likely also an exchange of ions (Boyle and Conway 1941) between the outer media and the cell interior leading to changes in the potential gradient across the membrane. It has been demonstrated (Stampfli and Nishie 1955, Schmidt and Stampfli 1959) that hypertonic media cause a decrease of the permeability of myelinated fibres to sodium and potassium and hypotonic media an increased potassium permeability. Similar changes may also occur in the end bulbs and in the afferent fibre and interfere with the depolarization caused by the mechanical distortion. There seems to be but little doubt, however, that the essential event underlying the action of anisotonic media on the spindle is the transfer of water through the sensory membrane. Teorell (1959 a, b, 1962) has developed an electrokinetic model of a mechanoreceptor which seems to have interesting bearings on the phenomena observed in the spindle. In this model excitation is suggested to occur as a result of a sequence of changes of the gradients of osmotic pressure, ionic concentration and electrical potential across the membrane, the most important change being the electro-osmotic flow of water through the membrane. It is not yet possible to determine to what extent the proposed functions of this electrohydraulic analogue are applicable to the events taking place in the sensory end bulbs. In the case of the muscle spindle several other factors may be involved and the final effect, as represented by the discharge and the change in sensitivity to stretch, can be assumed to be determined by a complex interplay of factors affecting a number of different functions and structures.

The author is greatly indebted to prof G. Hunt and prof C. Eyzaguirre for valuable suggestions in connection with the preparation of this paper

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The Effect of Small Doses of Chemically Pure Secretin on the Volume and Bicarbonate Output of the Pancreatic Juice in Dogs

By

H Säteri, L Thulin and B Welander

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Abstract

method Eight experiments were made in 2 unanesthetized dogs The doses ranged from 0 25 to 1 5 clinical units per kg b w and hr At the smaller doses there was only a slight increase of the volume and the bicarbonate output At the larger doses the maximum volume 25 ml per 30 min was about 3 times the initial and the maximum bicarbonate output 1 4—2 7 meq per 30 min about 50 times the initial in one dog and about 10 times in the other

Recently chemically pure preparations of secretin have been made available for studies of its effect on the pancreas and the liver Doses below 1 5 clinical units per kg b w and hr of the chemically pure secretin Secretin Vitrum (Jorpes and Mutt 1961, 1962) have no effect on the output of hepatic bile in dogs and the choleretic action of secretin in dog is thus much less than that of bile acids (Jonson and Thulin 1964)

The present study was undertaken in order to evaluate the effect on the pancreas in dogs of this chemically pure secretin at small doses without effect on the output of hepatic bile

Procedure

2 male mongrel dogs (dog A weight 16 kg and dog B weight 14 kg) were given 2 hr infusions of the chemically pure secretin Secretin Vitrum prepared by Jorpes and Mutt It contains 17 500 clinical units of secretin per mg substance

The pancreatic juice was collected according to Säteri (1963) A tube consisting of 2 tubes ... through one ... onger ... ined ... The ... juice ... The

TABLE I Mean pancreatic juice volume and bicarbonate output during basal experiments (exp 1) and during initial periods of secretin experiments (exp 2—4)

	Experiment			
	1	2	3	4
Volume, ml per 30 min				
Dog A	15 3	7 1	18 5	8 1
Dog B	14 6	9 3	9 8	8 0
Bicarbonate, meq per 30 min				
Dog A	0 245	0 138	0 191	0 055
Dog B	0 155	0 198	0 146	0 260

TABLE II Mean pancreatic juice volume and mean bicarbonate output during administration of secretin

	Dose, clinical units per kg bw and hr					
	0 25	0 50	0 75	1 00	1 25	1 50
Volume, multiples of initial						
Dog A	1 6	1 4	2 2	1 9	3 6	3 3
Dog B	1 4	1 4	2 2	3 0	2 6	3 3
Bicarbonate multiples of initial						
Dog A	1 7	5 1	16 9	18 0	7 6	49 0
Dog B	1 1	4 1	3 3	9 9	6 1	7 8

sterile saline with secretin were continuously infused a certain dose of secretin being given during each period These doses were 0 25 and 1 25 (exp 2), 0 5 and 1 0 (exp 3), 0 75 and 1 5 (exp 4) clinical units per kg b w and hr

The pancreatic juice of transitional stages i e the first half hour of each 2 hr period was omitted

Results

Basal experiments (Table I) Mean volumes during the three 2-hr periods were 17 3, 14 9 and 16 7 ml per 30 min (mean during the whole experiment 15 3) in dog A and 8 5, 18 5 and 17 0 (mean 14 6) in dog B

Mean bicarbonate outputs were 0 175, 0 278 and 0 281 meq per 30 min (mean during the experiment 0 245) in dog A and 0 109, 0 199 and 0 157 (mean 0 155) in dog B

Secretin experiments Mean volumes during the initial periods were, on the whole, smaller than the volumes in the basal experiments (Table I) Mean bicarbonate outputs

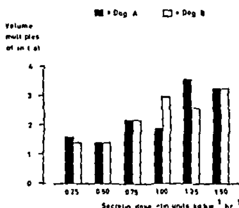


Fig 1

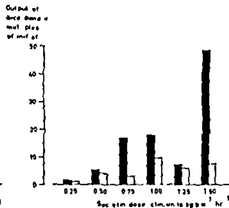


Fig 2

Fig 1 Volume of pancreatic juice during infusion of chemically pure secretin in two dogs
Fig 2 Output of bicarbonate during infusion of chemically pure secretin in two dogs

during the initial periods were smaller than the output in the basal experiment in dog A and equal or larger in dog B (Table I)

Mean volume during secretin infusion increased by increasing dose to 3 3 times the initial in both the dogs (Table II, Fig 1) The bicarbonate output increased by increasing dose to 49 times the initial in dog A and 7 8 times in dog B (Table II, Fig 2)

Discussion

The increase of the volume as well as the bicarbonate output was roughly proportional to the increase of the secretin dose The increase of the bicarbonate was more pronounced than that of the volume The increase of the volume at the dose 0 5 clinical units per hr and kg b w might be spontaneous as well as the increase of the bicarbonate output at 0 25 clinical units The largest increase of the bicarbonate output — nearly 50 times the initial — might depend on the low initial value during that experiment

A pancreatic duct fistula would be the most exact way to collect all the pancreatic juice It was shown by Sateri (1963), however that there is a fairly good agreement between the non surgical method used in the present study and a fistula method Thus, it seems that doses without effect on the output of hepatic bile have a significant effect on the output and composition of pancreatic juice in the dog The effect may, however, be weaker than in man, where a single dose of one unit gives a marked pancreatic response

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Effect of Potassium on the Movement of Water Across the Isolated Amphibian Skin

By

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Abstract

skin to potassium and vasopressin were observed

Bricker, Biber, and Using (1963) reported that replacement of most of the sodium by potassium in the Ringer solution bathing the inner surface of the isolated frog skin resulted in a reduction in the transepithelial potential difference and a relatively smaller reduction in the short-circuit current Vasopressin did not stimulate the short circuit current and the potential difference when the inner bathing solution was KCl Ringer, although a stimulation was seen when the inner solution was K_2SO_4-Ringer (Ussing, Biber and Bricker 1964, umpublished observations) Consequently, it was thought that it would be of interest to determine the effect of this high potassium treatment on the volume flow of water across the amphibian skin in the presence of an osmotic gradient A comparison of these effects with those of vasopressin has also been made

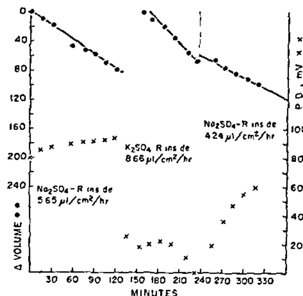


Fig 1. A representative experiment *which shows the effects of* substituting potassium for sodium in the inner solution on the transepithelial net water movement and potential difference The lines were fitted by inspection to the points relating change in volume of the outer solution to time The net water movements from the outer solution are calculated from the slopes of these lines The outer solution was composed of 1/10 Na_2SO_4-Ringer

Methods

inner solution was replaced by potassium For the determination of the potential difference across the skin bridges containing 3% agar agar in Ringer led to saturated KCl solution which was in contact with calomel electrodes The potential difference was measured with a Radiometer PHM4 potentiometer The surface area of the skin was 7 cm² in each experiment

An equilibration period of at least 45 min was allowed before measurements were begun There was usually an interval of 15 min between successive measurements The rate of net water movement from the outer solution to the inner solution was estimated graphically from the slope of the line relating the measured change in volume of the outer solution to time (Fig 1 and 2) For this purpose 4 to 6 successive measurements which showed minimal scatter around this line were usually required although in a few cases only 3 measurements were used In experiments in which there were relatively large net movements of water both inner and

at the end of this interval and measurements of water movement were begun

The initial experiments were performed on frogs which were brought into the laboratory in the fall and winter The responses to the high potassium treatment and to vasopressin were quite variable It was then found that uniform responses could be obtained with toads although the magnitude of the response varied with the season and method of storing the animals Most

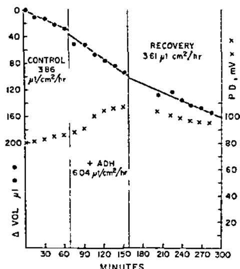


Fig 2 A representative experiment which shows the effects of the addition of vasopressin to the inner solution on the transepithelial net water movement and potential difference

of the experiments to be reported here were performed on toads which were brought into the laboratory in October, 1962 and stored at 20° C for 2 to 3 months They were subsequently transferred to the cold room where they were kept at 4° C for several weeks prior to use The last experiments with these toads which will be referred to as "Fall" toads were completed by the end of April 1963 The toads used in the subsequent experiments, "Spring toads arrived in the laboratory in April 1963 They were kept at 20° C without food until used

[illegible]

Results

The replacement of all of the sodium in the inner solution by potassium resulted in an increase in net movement of water from outer to inner solutions In the Fall toads the volume flow of water was increased by about one-third The relative response was [illegible]

considerably greater than in the Fall toads, approximately three and one half times the control rate of water movement (Table V A and VI A) When the major anion in the Ringer solution was sulfate, changing the inner solution back from K Ringer to Na Ringer resulted in an almost complete return of net water movement to control values (Table I A) Recovery in chloride Ringer was not as good (Table I C

[1] The osmotic pressure of sulphate Ringer is about 2 3 of that of chloride Ringer

TABLE I Comparison of effects of potassium and vasopressin (ADH) in the inner solution on the net water movement ($\mu l/cm^2/hr$) across the toad skin. The inner solution was Na Ringer in the control and recovery periods. The outer solution was 1/10 Na Ringer in all periods. Means ± S.E. The numbers in parentheses are the number of experiments. Fall toads.

I Control	II Experimental	III Recovery	II/I
A SO_4 ringer. Na of inner solution replaced by K in experimental period			
6.19±0.51	8.18±0.57*	6.25±0.68	1.36±0.09
(9)	(9)	(9)	(9)
B SO_4 ringer. ADH in inner solution in experimental period			
4.95±0.44	7.58±0.57*	4.99±0.42	1.61±0.17
(8)	(8)	(7)	(8)
C Cl ringer. Na of inner solution replaced by K in experimental period			
10.68±0.95	14.24±1.56*	13.10±1.13	1.32±0.04
(6)	(6)	(6)	(6)
D Cl ringer. ADH in inner solution in experimental period			
10.71±1.29	16.00±1.83**	11.65	1.54±0.20
(5)	(5)	(2)	(5)

Experimental period significantly different from control period at $p < 0.005$*, < 0.05**

TABLE II Comparison of effects of vasopressin (ADH) in Na_2SO_4 Ringer and in K_2SO_4 Ringer in inner chamber on net water movement ($\mu l/cm^2/hr$) across the toad skin. Outer solution was 1/10 Na_2SO_4 Ringer in all periods. Means ± S.E.

I Na Ringer	II ADH in Na Ringer	III ADH in K Ringer	IV Na Ringer	II/I	III/II
A Fall toads. 8 experiments					
5.79±0.72	7.58±0.42	8.24±0.69	6.19±0.88	1.50±0.27	1.08±0.04
B Spring toads. 8 experiments					
10.8±1.6	[1]40.8±7.4	42.1±7.8	21.0±3.6	3.99±0.73	1.08±0.16

[1] Significantly different from period I at $p < 0.01$

TABLE III Comparison of effects of K_2SO_4-Ringer and K_2SO_4-Ringer plus vasopressin (ADH) in inner chamber on net water movement (μl/cm²/hr) across the toad skin. Outer solution was 1/10 Na_2SO_4-Ringer in all periods Means ± S E Numbers in parentheses indicate number of experiments Fall toads

I	II	III	IV	II/I	III/II
Na Ringer	K Ringer	K-Ringer + ADH	Na Ringer		
5 01 ± 0 26 (6)	6 76 ± 0 74 (6)	7 24 ± 0 76 (6)	7 31 ± 1 62 (4)	1 36 ± 0 48 (6)	1 03 ± 0 26 (6)

TABLE IV Effect of low pH on response of toad skin to a high potassium concentration in inner solution. SO_4 Ringer inside, 1/10 Na_2SO_4 Ringer outside 5% CO_2 in air bubbled through inner and outer solutions during entire experiment Means ± S E., 7 experiments Fall toads

I Na Ringer	II K-Ringer	II/I
Net water movement, μl/cm²/hr		
4 41 ± 0 39	[1]5 34 ± 0 62	1 19 ± 0 08

[1] Significantly different from I at $P < 0.025$

When the inner solution was changed from sodium to potassium Ringer, there was a rapid fall in the potential difference across the skin (P D) Sufficient measurements were not made to determine this minimal P D with certainty There was then a slow, small rise in P D to a peak which averaged 26 % of control in SO_4-Ringer and 17 % in Cl Ringer (Fall toads) The P D then fell gradually until the inner solution was changed back to Na Ringer In this recovery phase of the experiments the P D rose steadily, although the P D had not returned to control levels after as long as two and one-half hours The recovery P D averaged 65 % of control in Na_2SO_4-Ringer and 40 % of control in NaCl Ringer (Fall toads) when observations were discontinued one to two and one-half hours after changing to Na-Ringer These effects as well as the changes in water movement are illustrated by the representative experiment shown in Fig 1

The increase in net transepithelial water movement in response to the addition of asopressin to the inside solution was consistently somewhat higher than the response o potassium, *averaging 50 to 60 % in Fall toads and 300—460 % in Spring toads* Table I B and D, II B, V B, VI B, Fig 2) As was the case with the response to potassium, the relative response to vasopressin was essentially the same in both sulfate and hloride Ringer (Table I B and D) Recovery of water permeability following the wash-

TABLE \ Effect of changes in calcium concentration in inner solution on changes in net movement of water ($\mu l/cm^2/hr$) in response to high potassium and to vasopressin (ADH) in inner solution Cl Ringer inside, 1/10 NaCl Ringer outside. Means ± S E Spring toads

I Control	II Experimental	III Recovery	II/I
A Na of inner solution replaced by K in experimental period			
1 [Ca] = 0 6 experiments			
21 4±1 5	76 5±8 5	34 0±6 4	3 65±0 41
2 [Ca] = 0 9 mM, 6 experiments			
18 5±2 1	58 1±3 5	27 4±3 0	3 45±0 42
3 [Ca] = 15 mM 5 experiments			
18 9±4 2	45 3±12.2	44 8±14 1	2 49±0 55
B ADH in inner solution in experimental period			
1 [Ca] = 0 6 experiments			
15 5±1 4	140±23	18 9±1 9	9 09±1 42
2 [Ca] = 0 9 mM, 6 experiments			
23 6±3 9	93 4±16 7	28 4±3 9	4 40±0 90
3 [Ca] = 15 mM 6 experiments			
19 3±1 9	99 6±20 2	21 0±3 7	5 58±1 39

ing out of the vasopressin was almost complete under all conditions tested A representative experiment is shown in Fig 2

Two types of experiments were performed in order to determine whether the responses to vasopressin and the high potassium treatment are additive In one a period in which the inner solution was composed of Na_2So_4 Ringer plus vasopressin was followed by a period with K_2SO_4 Ringer plus vasopressin as the inner solution (Table II) In the second type of experiment a period with K_2SO_4 Ringer inside was followed by one with K_2SO_4 Ringer plus vasopressin (Table III) The response to the combination of high potassium plus vasopressin was on the average, 8 % greater than the response to either treatment alone However, this difference was small and not statistically significant, Thus the two effects are not additive

It has been shown that reducing the pH of the inner solution to 6 5 completely blocks the action of vasopressin on the toad bladder (Bentley 1958 Schwartz *et al* 1960) The effect of low pH on the changes in net water movement of the toad skin in response

TABLE VI Effect of changes in calcium concentration in outer solution on changes in net movement of water (μl/cm²/hr) in response to high potassium and to vasopressin (ADH) in inner solution SO_4-Ringer inside, 1/10 Na_2SO_4 Ringer outside Means ± S E Spring toads

I Control	II Experimental	III Recovery	II/I
Na of inner solution replaced by K in experimental period			
1 [Ca] = 0, 5 experiments			
9 13±0 55	31 8±6 6	19 3±6 0	3 52±0 75
2 [Ca] = 10 mM, 5 experiments			
7 65±0 9	25 4±4 8	12 1±1 8	3 54±0 89
B ADH in inner solution in experimental period			
1 [Ca] = 0, 5 experiments			
13 3 ±2 9	63 0±6 5	–	5 26±0 72
2 [Ca] = 10 mM, 6 experiments			
11 8 ±2 7	44 9±6 6	[1]20 1±8 8	4 26±0 70

[1] 4 experiments

to high potassium treatment and to vasopressin was investigated *Five per cent* CO_2 in air was bubbled through both the inner and outer solutions, reducing the pH in the inner solution to 6 3—6 5 and in the outer solution to 5 0—5 3 In 2 expts vaso-
[illegible] to the inner solution was without effect. The high potassium treatment
[illegible] toad skin by 19 % (Table IV) The
[illegible] obtained at normal pH (Table I
[illegible]

evidence that calcium [illegible] and Edelman 1962) Bentley (1959) has also presented evidence [illegible] is required for the action of vasopressin upon this tissue For these reasons a study was made of the effects of changing the calcium concentration in both inner (Table V) and outer (Table VI) solutions on the responses to vasopressin and to the high potassium treatment In those experiments in which the concentration of calcium in the inner solution was varied the major anion was chloride The inner Ringer solutions, containing calcium at either 0 mM (no added calcium) or 0 9 mM also contained sucrose

(45 mM and 42.4 mM, respectively) so that the osmotic pressures of these solutions were equivalent to the Ringer containing 15 mM calcium. The outer solution was the usual NaCl Ringer diluted ten fold and without added sucrose (final calcium concentration was 0.09 mM in all experiments). A change of the calcium concentration in the inner solution from 0 mM to 15 mM was without statistically significant effect on the responses to either potassium or vasopressin (Table V). The combination of high potassium and 15 mM calcium appeared to damage the skin irreversibly. The potential difference across the skin fell much more rapidly than in those experiments in which the calcium concentration was 0.9 mM or 0 mM. The change in net water movement was poorly reversible in the recovery periods when the inner solution was changed from K Ringer to Na Ringer, the potential stabilized at −5 to −8 mV in four out of the 5 expts.

Two experimental conditions were employed to study the effects of changes in the concentration of calcium in the outer solution: zero calcium (no added calcium, 0.5 mM sodium ethylene-diaminetetraacetate, 20 mM sucrose) and 10 mM calcium. There were no statistically significant differences in response to either potassium or vasopressin at these two calcium concentrations (Table VI).

It should be noted that changes in calcium concentration in either the inner or outer solutions were without effect on the water permeability of the toad skin under control conditions (Table V and VI). It was also found in both of these series of experiments that the response to potassium was significantly less than the response to vasopressin ($p < 0.05$ in the experiments in which the inner calcium concentration was varied; $p < 0.025$ in the experiments in which the outer calcium concentration was varied).

Discussion

It appears reasonable to consider that the increased net water movement which occurred when potassium replaced the sodium in the inner solution was the result of an increased permeability of the toad skin to water. However, Reid (1892) demonstrated a slight net transfer of water across the frog skin when it is exposed to Ringer solution on both sides. While it is possible that the high potassium treatment enhanced this phenomenon, it appears to be unlikely.

Certain aspects of the data which have been presented suggest the possibility that vasopressin and potassium act on some common mechanism to increase the permeability of the amphibian skin to water. First, the responses to these agents are not additive. Second, both agents act when in contact with the inner surface of the toad skin. While it has been clearly demonstrated that vasopressin increases the permeability of a permeability barrier at the physiological outer surface of the amphibian skin and urinary bladder (MacRobbie and Ussing, 1961; Hays and Leaf, 1962), evidence relative to the locus of action of potassium is not available. Third, seasonal changes in the responsiveness of the amphibian skin to vasopressin are accompanied by parallel changes in the responsiveness to potassium.

On the other hand, a reduction in the pH of the inner solution completely blocks the action of vasopressin, but, at most, only partially inhibits the response to potassium. However, it has been postulated that the first step in the action of vasopressin is its binding to the cell membrane (Rasmussen et al., 1960, 1963; Schwartz et al., 1960). Evidence has been presented that an elevated hydrogen ion concentration may interfere

with this binding (Schwartz *et al* 1960) Thus, it is possible that, if vasopressin and an elevated potassium concentration trigger" the same mechanism, the potassium acts at some intermediate point the in sequence between the binding of the hormone to the membrane and the change in membrane permeability However, the evidence for this concept is still highly circumstantial, so that one must consider the alternative possibility that vasopressin and potassium increase the permeability of the toad skin to water by different mechanisms Should this be the case, then it is however, necessary to postulate that a high concentration of potassium in the inner solution inhibits the action of vasopressin, since the two effects are not additive Some support for this possibility is found in the observation by Bentley (1959) that raising the concentration of potassium in the serosal medium to 10 mM decreases the water permeability of the isolated toad bladder in the presence of vasopressin Ussing, Biber and Bricker (1964, unpublished observations) have shown that vasopressin does increase the short-circuit current in frog skins with K_2SO_4 Ringer on the inside, although there is no effect if the inner solution is KCl Ringer Thus potassium ions do not inhibit all effects of vasopressin It was rather suggestive that the increase in current induced by vasopressin in K_2SO_4 treated frog skins was associated with a considerable swelling of the epithelium In the absence of vasopressin the epithelial volume remained constant for at least one hour if sulfate was the anion, whereas there was spontaneous swelling and increase in short circuit current if chloride was the main anion One might therefore venture the hypothesis that high potassium increases the permeability of the outer diffusion barrier of the skin to water as well as to sodium by bringing about swelling of the epithelial cells The absence of a vasopressin effect on the current with KCl on the inside would be understandable if we assume that the swelling in KCl in itself gives near maximum effect on the sodium permeability If a similar explanation were valid for the effects on water permeability, it would imply that the epithelial cells of toad skins swell in K_2SO_4 as well as in KCl Ringer This question is as yet open since the wrinkled surface of the toad skin makes it impossible to measure epithelial volumes according to MacRobbie and Ussing (1961) Quite generally, osmotic swelling of the frog skin epithelium leads to increased active sodium transport and shrinkage to inhibition of the transport (Ussing 1964) If vasopressin increases the entry of sodium in the cells, it would lead to swelling which in turn might also lead to increased water permeability

There is some disparity between the observations of Bentley (1959) and those presented here concerning the effects of changes in calcium concentration on the response to vasopressin Bentley found that a reduction in calcium concentration reduced the water permeability of the toad bladder in the presence of vasopressin, in our experiments such an effect was not observed However, Bentley omitted the calcium from the solutions bathing both sides of the bladder, and there may also be qualitative differences in the responsiveness of the two tissues We have also failed to observe the diminution in response to vasopressin at elevated serosal calcium concentrations as reported by Bentley However, the concentrations of vasopressin was 1 mU/ml in Bentley's experiments and 87 mU/ml in our experiments Petersen and Edelman (1962) reported that increasing the vasopressin concentration to 66—100 mU/ml abolished 8 % of the inhibitory effect of the elevated calcium concentration

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Monoamines in the Swimbladder of Gadus callarias and Salmo irideus

By

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Abstract

Fahlén G, B. Falck and E. Rosengren. *Monoamines in the swimbladder of Gadus callarias and Salmo irideus.* Acta physiol. scand. 1965. 64. 119—126. — The noradrenaline in the swimbladder of the cod and the rainbow trout is stored in short adrenergic neurons whose perikarya are located in close relation to the swimbladder itself. In the cod the noradrenergic neurons are innervated by noradrenergic neurons that arise from the coeliac ganglion of the sympathetic chain. A hitherto unknown system of epithelial cells storing 5-hydroxytryptamine was observed in the trout swimbladder.

Substantial evidence was presented by Fänge (1953) that the muscular fibres of the secretory part of the cod swimbladder, together with the radial muscle of the oval edge, are sensitive to adrenaline (A) and noradrenaline (NA). Euler and Fänge (1961) found that the muscularis mucosae and the gas gland in the swimbladder of the cod contained much greater amounts of NA than of A. Indeed, the amount of NA was of a magnitude that could well correspond to an adrenergic innervation of smooth musculature in the swimbladder. However, after denervation of the swimbladder no decrease of the NA content was observed, and it could not therefore be excluded that the NA in the swimbladder might be stored in places other than the adrenergic nerves, presumably in some kind of chromaffin cells. This assumption is interesting since it might imply a release of transmitter — in this case NA — from chromaffin cells. Such an organization was assumed to be a general principle in the adrenergic system by Burn and Rand (1960) and Brandon and Rand (1961) who suggested that NA is not localized in adrenergic neurons but in e. g. chromaffin cells. This theory contradicts the reports of Euler's investigations (cf. Euler 1956, 1961) which offer strong evidence that this amine is stored in the mammalian adrenergic neuron, principally in its terminal part. The accuracy of Euler's concept was directly demonstrated recently by Falck and Torp (1962) and Falck (1962).

It is thus of considerable importance to obtain more accurate knowledge of the storage site of NA in the swimbladder This is now made possible by the introduction of a highly sensitive and specific fluorescence method for the detection of certain catecholamines and tryptamines at the cellular level (Falck and Torp 1961, Falck 1962, Falck *et al* 1962 Falck and Torp 1962)

Material and methods

The histochemical method is based on the principle that the catecholamines and 5-hydroxytryptamine (5-HT) can condense with formaldehyde to produce intensely fluorescent products in the tissues. Provided that the reaction takes place under fairly dry conditions no diffusion of the amines from their cellular localization will occur Usually the tissues to be analyzed must be freeze-dried Thin tissue sheets however can be dried for a short time at room temperature *in vacuo* usually without any diffusion of the amines. In fish swimbladder the last method may cause diffusion This however can be avoided by freeze-drying the stretch preparations of the tissue sheets.

Gas glands of the swimbladder of the cod *Gadus callarias*, including the distal part of nerves and vessels entering the swimbladder in the centre of the gas gland region, and also the coeliac ganglion and the splanchnic nerve were freeze-dried treated with formaldehyde gas for 1 hr at 80 C or 1 5 hrs at 60 C, and then infiltrated with paraffin *in vacuo* Sections (8 μ) were mounted in Entellan Merck) and examined under the fluorescence microscope in dark field illumination Pieces from different regions of the swimbladder of the rainbow trout *Salmo irideus* were treated in the same way

Thin sheets of the oval edge that separates the secretory and the resorbent parts of the swimbladder of the cod were carefully stretched over a plastic ring freeze-dried, and mounted in Entellan after a short time in xylene. The technique is described in detail elsewhere (Falck 1964 Falck and Owman 1964)

For the catecholamine and 5-HT analyses whole swimbladders of the rainbow trout were weighed and minced with 0 4 N perchloric acid. Dopamine (DA) NA and A were purified according to Bertler *et al* (1958) and determined as described by Häggendal (1963) 5-HT was



injection

Results

Cod In the gas gland region and in the stretch preparations of the oval edge of the cod swimbladder a specific moderate green fluorescence developed in typical nerve bundles consisting of smooth fibres An intense green fluorescence developed in fine varicose fibres some of which were clearly seen to issue from the nerve bundles and thus obviously constituted the terminal parts of the neurons. An abundance of varicose fibres was present in the gas gland and especially in the radial muscle of the oval edge, numerous nerves also ocurred in the rete mirabile and the muscularis of the secretory part and around the vessels In the wall of the resorbent part of the swimbladder the fluorescent fibres were much more sparse and seemed to be distributed to the vessels only No fluorescent cells were observed Nonfluorescent ganglion cells were found scattered at the base of the vascular bundles forming the rete mirabile The fine varicose green fluorescent fibres of the gas gland proper the rete mirabile and the muscularis were seen to originate from moderately green fluorescent ganglion cells situated immediately outside the swimbladder wall in the bundle of nerves and vessels



[illegible] immediately outside the swimbladder [illegible] and region. Moderately [illegible] [illegible]minal fibres are seen [illegible] Magnification 675

entering the organ in the centre of the gas gland region The smooth preterminal fibres from these ganglion cells were collected in a thick bundle accompanying the vessels into the wall Beyond the entrance this nerve bundle divided into several small branches Some of them passed directly into the rete mirabile the gas gland, and the muscularis of the secretory part, where they gave rise to the terminal varicose fibres Other bundles accompanied the veins coming from the resorbent part to the oval edge

The swimbladder is sympathetically innervated by branches from the right splanchnic nerve which arises from the first 2 sympathetic ganglia. These ganglia often — as in the cod — constitute one single ganglion called the *ganglion c[illegible]* Stannius 1849 In the present investigation this ganglion proved to be composed of relatively large ganglion cells emitting a weak green fluorescence non fluorescent cells could not with certainty be detected. Scattered among the ganglion cells was found a peculiar type of cells irregular in form that gave off short slender processes running in between a few of the contiguous nerve cells These small cells exhibited a very stron[g] green to yellow [g]reen fluorescence well comparable to that found in adrenal medullary cells The same type of cells was found to occur in mammalian ganglia Hamberger *et al* 1963 Falck *et al* 1964 The weakly fluorescent preterminal fibres from the ganglion

TABLE I Contents of NA (μg/g) in the swimbladder of the cod

Number of exp	Gas gland	Oval mucosa	The peripheral ganglion outside the gas gland
1	0.46	1.50	0.80
2	0.53	0.63	0.29
3	0.49	1.30	0.42

cells assembled to form the splanchnic nerve. Along its course, a small amount of scattered, fluorescent ganglion cells was observed. Most of the fibres in this nerve go to peripheral ganglia in the gut wall, others are directed towards the swimbladder (Young 1931).

The last-mentioned neurons transformed into highly fluorescent varicose fibres within the adrenergic ganglion situated contiguous to the swimbladder wall synaptically enclosing the fluorescent cell bodies in this ganglion (Fig. 1).

The amount of NA in the different parts of the swimbladder is given in Table I. The concentrations correlate well with the fluorescence microscopic findings, except for the values found in the peripheral ganglion outside the gas gland. Considering the number of nerve cells and the fluorescence intensity, which was unusually high in the preterminal part of an adrenergic neuron, the concentration found seems to be too low. However, this can be explained by the fact that the ganglion could not be entirely freed from surrounding tissue. In three determinations the coeliac ganglion was found to contain 7.6 μg/g (4.7—9.2) catecholamine, consisting mainly of NA. It could not be excluded that a small proportion represented A. It is in this connection interesting to note that the small, highly fluorescent cells in mammalian ganglia may contain A (Owman and Sjöstrand 1965). The NA values found seem to agree well with the histochemical findings and with the NA level in adrenergic ganglia found by other investigators (cf. Muscholl and Vogt 1958).

Rainbow trout. It seemed of great interest to study the adrenergic innervation apparatus also in a species with a swimbladder of the physostomous type — that is, with an open connection to the oesophagus and without a distinct gas gland. The rainbow trout, *Salmo irideus*, was chosen for this purpose. In the anterior part of the swimbladder, which in the form of a narrow duct connects the organ with the oesophagus, were observed a moderate number of nerve cells of varying size showing a rather weak green fluorescence. The cells appeared in the connective tissue layer, outside the muscularis, scattered or in groups. Some of the cells were situated in the vicinity of the muscle layer, and in these instances the whole neuron could often be observed. In such neurons the first part of the axon appeared smooth and rather weakly fluorescent, but when the axon entered the muscle layer, its character altered: it became transformed into a varicose fibre that showed a much more intense fluorescence. From the ganglion cell groups, the axons traversed to the muscle layer collected in slender nerve bundles from which varicose fibres issued at the periphery of the muscularis. A very considerable number of varicose fibres were found in the muscularis arranged parallel to the circular muscle fibres. The occurrence of these fibres was extremely rich, suggesting that every



Fig 2 Cod swimbladder Stretch-preparation of the oval edge Adrenergic fibre bundles and varicose terminal fibres Magnification 675 ×

muscle fibre made contact with one or more axons Only occasional fibres passed into the subepithelial connective tissue layer In other parts of the swimbladder the adrenergic innervation of the muscularis was the same as in the anterior part but here no fluorescent ganglion cells were found

The chemical analyses showed the presence of NA in a mean concentration of 0 33 μg g (0 26—0 43) in the swimbladder of the rainbow trout A and DA were not found in any significant amounts in that organ

An interesting finding was that a yellow fluorescence developed in ovoid cells located in the epithelium of the swimbladder These cells were generally provided with thin processes one apical reaching the epithelial surface and one or several reaching the base of the epithelium These processes could be clearly observed because they also contained strongly fluorescent material Their basal processes generally terminated in small enlargements The fluorescent reaction in these cells suggests that they may contain a tryptamine This agrees well with the results obtained from the chemical analyses which showed the presence of significant amounts of 5-HT 0 50 μg g (0 40—0 68

After treatment with reserpine no fluorescence or only a faint one was seen in the neurons and only few weakly yellow fluorescent cells remained in the epithelium The fluorimetric determinations revealed a considerable reduction of the 5-HT content (0 10—0 18 μg g and an almost complete depletion of NA



Fig 3 Rainbow trout swimbladder Transverse section of the wall of the anterior part demonstrating the abundance of varicose fibres in the circular muscle layer An adrenergic nerve cell body is seen in the surrounding connective tissue layer Magnification 750 ×.

Discussion

The high sensitivity and specificity of the present method for the catecholamines and 5-HT are discussed at length by Falck (1962 1964) Falck *et al* (1962) and Corrodi and Hillarp (1963 1964 The reaction conditions under which the green fluorescence developed in neurons in the cod and trout tissue specimens, and the properties of the fluorescent product indicate that a primary catecholamine is demonstrated Adrenaline-containing neurons need a much longer exposure to formaldehyde gas to develop a maximal fluorescence (Falck *et al* 1963) Only cells that store huge amounts of A, *i e* chromaffin cells in the adrenal medulla and in other locations, show up under the reaction conditions used in this investigation (*cf* Falck and Torp 1961 Dahl *et al* 1964, Owman and Sjostrand 1964 The only catecholamine found spectrophotofluorimetrically to be present in significant amounts was NA The histochemical criteria likewise indicate that the yellow fluorescent cells in the trout swimbladder epithelium contain a tryptamine derivative No other tissue structure developed this type of fluorescence Spectrophotofluorimetrically the trout swimbladder was found to contain significant amounts of 5-HT Furthermore, after reserpine treatment a considerable decrease in

green and yellow fluorescence was observed, and no NA and only slight traces of 5-HT could be found fluorimetrically Thus there seems no doubt that the green neuronal fluorescence demonstrated NA and that the yellow fluoresence was derived from 5-HT stored in the special epithelial cells

The morphology of the nervous structures in the swimbladders of the cod and trout was the same as in the adrenergic autonomous system of mammals (Hillarp 1959, Falck 1964) The distribution of the fluorescent material agrees well with the distribution of the adrenergic transmitter in mammalian adrenergic nerves (*cf* Euler 1961, Falck 1962)

The finding of numerous adrenergic neurons located entirely peripherally explains well the result of the earlier denervation experiments The possibility that the cod swimbladder contains chromaffin cells whose chromaffinity is due to a storage of catecholamines can be disregarded since no fluorescent cells — except ganglion cells — were found in the swimbladder and since the method used has a sensitivity of quite another magnitude than the chromaffin reaction

The finding that the adrenergic neurons which arise in the coeliac ganglion store NA is surprising considering the fact that they terminate synaptically around the peripheral noradrenergic perikarya. Many investigations have demonstrated adrenergic fibres synaptically terminating upon adrenergic cell bodies in mammalian sympathetic ganglia (Falck 1962, Hamberger and Norberg 1963 Hamberger *et al* 1963, Falck *et al* 1964, Owman and Sjostrand 1964) and there is support for the assumption that noradrenergic neurons are linked together in such ganglia (Hamberger *et al* 1963)

The physiological significance of the 5-HT-containing cells in the trout swimbladder is not known Morphologically these cells have the same appearance as the chromaffin cells in several mammalian species that also carry 5-HT and are provided with apical as well as basal processes (Falck, unpublished observations

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Occurrence of Dihydroxyphenylalanine Decarboxylase in Nerves of the Spinal Cord and Sympathetically Innervated Organs

By

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Abstract

sympathectomy reduced the dihydroxyphenylalanine decarboxylase activity in the rat submaxillary gland and spleen by 85 and 80 per cent respectively

The enzyme L-3 4-dihydroxyphenylalanine (DOPA) decarboxylase was first demonstrated in the guinea pig kidney (Holtz Heise and Ludtke 1938) It catalyzes the formation of dopamine (DA) from DOPA and is now generally accepted to be involved in the biosynthesis of catecholamines as was first proposed by Blaschko (1939 Later on *strong evidences have been put forward for the view that the DOPA decarboxylase is* identical with the L-5-hydroxytryptophan decarboxylase the corresponding enzyme in the biosynthesis of 5-hydroxytryptamine (5-HT) Westermann Balzer and Knell 1958 Yuwiler Geller and Eiduson 1959 Bertler and Rosengren 1959 a Rosengren 1960 One of the the principal functions of the catecholamines and 5-HT in the body seems to be serving as humoral transmitters of certain central as well as peripheral neurons The DOPA decarboxylase is however widely distributed in the organism

and, actually, most of the enzyme does not appear to be localized in neurons Until recently the relatively low activity of DOPA decarboxylase in the sympathetic ganglia and postganglionic trunks (Holtz and Westermann 1956) was the only evidence for the presence of the enzyme in nerves In a preliminary communication we have reported that the DOPA decarboxylase activity almost completely disappears from the spinal cord caudally to a transection and from the spleen after postganglionic sympathectomy (Andén, Magnusson and Rosengren 1964) These findings prompted the present investigation into the time courses of the disappearance of the enzyme and noradrenaline (NA) after lesion of central as well as peripheral monoamine neurons

Experimental

Adult rabbits of either sex weighing 2—3 kg were used The spinal cord was transected at the level of Th 4—Th 6 This procedure like all other surgical operations in this investigation was carried out under pentobarbital sodium anesthesia Care was taken to prevent hypothermia residual urine, decubitus and wound infections postoperatively In order to estimate the effect of the lesion on the DOPA decarboxylase activity *in vivo* the DA content of the cord was determined after administration of L-DOPA (100 mg/kg i v) The animals were sacrificed by air embolism 60 min after the DOPA administration The spinal cord was carefully freed from the meninges and nerve roots The DOPA decarboxylase activity *in vitro* was determined in pieces of the thoracic cord 2 5—3 0 cm in length (0 2—0 6 g) cranially and caudally to the lesion The pieces were taken from the animals used for the DOPA decarboxylase determinations *in vivo* and were rapidly put into phosphate buffer instead of perchloric acid The extraction of the DOPA decarboxylase and the incubation with L-DOPA were performed according to Bertler and Rosengren (1959 b) The DA formed *in vitro* as well as *in vivo* was estimated by the method of Carlsson and Waldeck (1958) as modified by Carlsson and Lindqvist (1962) The NA was determined as described by Bertler Carlsson and Rosengren (1958) Control values were obtained from animals not operated on

In other experiments the effect of sympathetic denervation on the DOPA decarboxylase activity in the rabbit iris was studied The left superior cervical ganglion and the continuous sympathetic chain cranially to the clavicle were removed After various intervals of time the animals were sacrificed by air embolism The DOPA decarboxylase activity of the right and the left iris was determined *in vitro* as described above The iris was preganglionically sympathectomized by excision of 3—4 cm of the left cervical sympathetic chain beginning about two cm caudally to the superior cervical ganglion

The DOPA decarboxylase activity in the denervated rat submaxillary and sublingual glands was also determined For reasons presented below it was necessary to ligate the ducts bilaterally when the enzyme activity in this tissue was to be determined The ligation was performed near the hilus On the same occasion the left superior cervical ganglion with the continuous sympathetic chain cranially to the clavicle were removed The operation was carried out under a Zeiss operation microscope The rats were sacrificed by a blow on the neck 14—16 days after the operation The right and left submaxillary glands including the sublingual glands were analysed separately for DOPA decarboxylase activity *in vitro* as described above The enzyme activity of the gland on the right side was used as a control value

The rat spleen was postganglionically sympathectomized by the excision of the celiac ganglion and as much as possible of the left and right splanchnic nerves as well as the soft tissue surrounding the celiac and superior mesenteric arteries A Zeiss operation microscope was employed After ten days or more the animals were decapitated The DOPA decarboxylase activity of the spleen *in vitro* was estimated as described above Control values were obtained from animals not operated on

Results

DOPA decarboxylase activity of the spinal cord The DOPA decarboxylase activity of the cord cranially to the section level was found to be 77 μg/g expressed as the DA formed *in vitro* after incubation with L DOPA for 45 min (S E 5 2 μg/g, 26 expts The enzyme

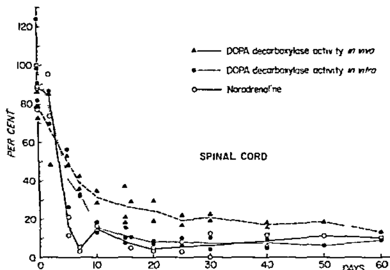


Fig 1 The dihydroxyphenylalanine (DOPA) decarboxylase activity and the noradrenaline concentration in the caudal part of the rabbit spinal cord at various intervals after transection Each symbol represents one single determination. *Ordinate* enzyme activity and noradrenaline concentration in the part caudally to the lesion in per cent of that cranially

activity of this part of the cord in the operated animals did not differ significantly to that found in animals not operated upon As DA does not occur normally in measurable amounts in the spinal cord the DA formed *in vivo* after injection of L-DOPA can also be used as a measure of the DOPA decarboxylase activity The DA found in the part cranially to the transection 60 min after an i v injection of 100 mg/kg L-DOPA was 2 1 µg/g (S E 0 14 µg/g 26 expts) Nor did the values from the controls and the animals operated on differ significantly in these experiments. The activities of the DOPA decarboxylase in the cord showed large individual differences when determined *in vitro* as well as *in vivo* It was found, however, that in the non-operated animal the activities were about the same in pieces taken cranially and caudally to the transection (see Fig 1 As the transection had no apparent effect on the activity cranially to the lesion the activity of the DOPA decarboxylase in the caudal part is given as per cent of that found in the cranial part It was found that transection caused a considerable reduction of the DOPA decarboxylase activity in the caudal part of the cord as determined *in vitro* as well as *in vivo* (Fig 1 The drop of the DOPA decarboxylase activity was most marked between the second and fifth day but a gradual reduction of the enzyme activity was found to occur during the following two weeks After that time the enzyme activity was not further reduced, it reached a constant level corresponding to 5—10 and 15—25 per cent of the cranial one as determined *in vitro* and *in vivo* respectively

The NA dropped in the caudal part of the spinal cord from section Fig 1 as described earlier Andén *et al* 1964 The NA level cranially to the lesion was 0 26 µg g S E 0 018 µg g 24 expts 60 min after the injection of L-DOPA 100 mg kg i v) There was no significant change of the NA content in this part of the cord after opera

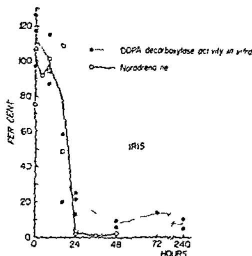


Fig 2 The dihydroxyphenylalanine (DOPA) decarboxylase activity *in vitro* and the noradrenaline content in the rabbit irides at various intervals after the excision of the superior cervical ganglion Each symbol ... units

tion Caudally the NA concentration was ...
cran ...
th ... three days the NA level showed a
ste... after 7 days the NA had almost completely disappeared in the caudal
part

DOPA decarboxylase activity of the iris The DOPA decarboxylase activity in the iris of the right side (i e of the side not operated upon) was 5 5 μg/iris (S E 0 47 μg/iris, 14 experiments) expressed as the amount of DA formed *in vitro* after incubation with L-DOPA for 45 min Between 8 and 16 hrs after the excision of the left superior cervical ganglion the decarboxylase activity of the left iris substantially dropped (Fig 2) The minimum level was reached after about 48 hrs when only approximately 10 per cent of the original activity was observed The values of the NA in the iris after postganglionic sympathectomy (Fig 2) are taken from a previous investigation (Andén *et al* 1964) Sixteen hours after the operation almost all of the NA remained while during the following 8 hrs it disappeared almost completely During the NA disappearance there was a distinct mydriasis on the sympathectomized side

The decarboxylase activity in the iris ten days after preganglionic sympathectomy on the left side was assayed in two experiments The activities of the left iris were 97 and 94 per cent of those of the right one

The postganglionic nerve trunks immediately adjacent to the superior cervical ganglia were analyzed for their DOPA decarboxylase activity in 2 expts on normal rabbits and compared to those found in the irides and the ganglia of the same animals The DA formation *in vitro* in the nerve trunk extracts was 860 and 620 μg/g while it was 57 and 33 μg g in the iris extracts i e the decarboxylase activity was 15—20 times higher at the former site The DOPA decarboxylase activity in the superior cervical ganglia of these animals was 4 900 and 5,600 μg/g, i e 5—10 times higher than that in the postganglionic trunks

DOPA decarboxylase activity of the rat submaxillary gland It was not possible to detect any DOPA decarboxylase *in vitro* in the normal submaxillary gland It was also found

TABLE I The dihydroxyphenylalanine (DOPA) decarboxylase activity of the rat submaxillary plus sublingual glands. The enzyme activity was determined in vitro and is expressed as μg dopamine formed per g tissue after incubation with L DOPA for 45 min The left superior cervical ganglion was removed 14—16 days earlier On the same occasion the ducts of the glands were ligated bilaterally

	Number of experiments	DOPA decarboxylase activity (mean ± S E)	Difference
Right (=control) side	5	101 ± 14 7	p < 0 001
Left (=denervated) side	5	16 ± 3 0	

TABLE II The dihydroxyphenylalanine (DOPA) decarboxylase activity of the rat spleen The enzyme activity was determined in vitro and is expressed as μg dopamine formed per g tissue after incubation with L-DOPA for 45 min In the denervated group the celiac ganglion was excised 10—46 days earlier Nonoperated rats were used as controls

	Number of experiments	DOPA decarboxylase activity (mean ± S E)	Difference
Controls	6	8 2 ± 0 96	p < 0 001
Denervated	6	1 7 ± 0 09	

that no DOPA decarboxylase activity of a brain extract which if incubated alone showed a rather high DA formation could be demonstrated if it was incubated together with an extract from an intact submaxillary gland However there was a clearcut DOPA decarboxylase activity in extracts from submaxillary glands which had atrophied in consequence of duct ligation about 14 days earlier (Table I) Further, there was no reduction of the DOPA decarboxylase activity in an extract from brain when incubated together with an extract from a gland duct ligated After the excision of the superior cervical ganglion the DOPA decarboxylase activity in the duct ligated gland was reduced by some 85 per cent about 14 days later (Table I) The corresponding NA loss amounted to more than 95 per cent

DOPA decarboxylase activity of the rat spleen The DOPA decarboxylase activity was determined in vitro in extracts from the rat spleen 10—14 days after the excision of the celiac ganglion The operation caused a drop of the enzyme activity by on the average 80 per cent Table II The NA level was reduced by 80—95 per cent after the same operation

Discussion

It is probable that the DOPA decarboxylase is present in monoamine nerves both in the central nervous system and in the periphery as after the degeneration of these nerves the organs studied in this investigation showed an almost complete disappearance of this enzyme activity The functional disturbance which occurs after cutting the nerve cannot be the cause as preganglionic sympathectomy of the iris did not produce the same effect To our knowledge the present investigation is the first to show that an enzyme participating in the metabolism of catecholamines and 5-HT is present at the mentioned sites Since long nerve degeneration experiments have indicated that the NA in the peripheral organs is localized in sympathetic nerve endings (Euler and Purkhold 1951, Goodall 1951) This view has been found to be true by means of the fluorescence method for histochemical demonstration of certain catecholamines and tryptamines (Falck 1962) Recently, investigations using this technique combined with biochemical studies and transection experiments have proved that the NA and 5-HT of the spinal cord are situated in descending nerve tracts (Carlsson *et al* 1964, Magnusson and Rosengren 1963, Carlsson, Magnusson and Rosengren 1963) There is, however, a marked difference in the time course of the disappearance of the NA in a peripheral organ and that of the monoamines in the spinal cord after denervation The depletion of the NA from the rabbit iris is completed within 24 hrs after the removal of the superior cervical ganglion, whereas the drop of the NA and 5 HT in the rabbit spinal cord occurred 2—7 days after the transection (Andén *et al* 1964) It is noteworthy that the disappearance of the DOPA decarboxylase from the spinal cord and the peripheral organs after denervation apparently showed a similar difference, the enzyme activity in the iris had reached its minimum within 48 hours whereas at the same interval of time it was on the whole unchanged in the spinal cord The disappearance of DOPA decarboxylase and NA followed roughly similar time courses after denervation but the enzyme did not seem to vanish as rapidly and suddenly as the amine The relatively high value of the enzyme activity in the caudal part of the spinal cord five days after transection may be explained by the apparently somewhat slower degeneration of the axons belonging to the 5 HT than to the NA neurons (Andén *et al* 1964) However, in the spinal cord it was evident that the last part of the enzyme was lost much later than NA and 5 HT which may, at least partly, be due to the barrier between the central nervous system and the blood

Also after the section of cholinergic nerves the amine (acetylcholine) and the enzyme responsible for its formation (choline acetylase) seem to disappear from the innervated structures Hitherto only the cholinergic nerve terminals in the sympathetic ganglia have been investigated in this respect The choline acetylase activity of the superior cervical ganglion falls almost to zero after removal of part of the cervical sympathetic chain (Feldberg 1943 Banister and Scrase 1950, Hebb and Waites 1956) The time course of the loss of the choline acetylase activity largely paralleled that of acetylcholine found by Mac Intosh (1938) and Feldberg (1943) The losses of [illegible] ganglia appear to occur [illegible] iris with [illegible]

There is strong evidence for the view that NA occurs everywhere in the adrenergic postganglionic neurons but that it is highly concentrated in the terminals present in the organs (Euler 1956 p 136, 1959 Falck 1962) The same conditions seem to prevail in

the central neurons storing NA, DA and 5 HT (Dahlström and Fuxe 1964) as well as in the peripheral neurons containing acetylcholine and choline acetylase (for references, see Hebb 1963). As mentioned above the DOPA decarboxylase in the nervous system was first detected in the non-terminal parts of the postganglionic sympathetic neurons (Holtz and Westermann 1956). Our finding that there is a factor of only 15—20 between the enzyme activity in the nerve trunk and in the iris indicates that the decarboxylase has a similar intraneuronal distribution as NA. This statement is based on the assumption that the sympathetic nerves in the iris like in the spleen amount to not more than 1/1,000—1/10,000 of the organ weight (Euler 1956 p. 136). The somewhat higher decarboxylase activity in the superior cervical ganglia than in the postganglionic trunks may, at least partly, be due to the presence of noradrenergic nerve terminals at the former site recently discovered (Hamberger and Norberg 1964).

The figures obtained for the DOPA decarboxylase activity in the caudal part of the spinal cord after transection were found to be lower if the determination was performed *in vitro* than *in vivo*. The reason for the difference may possibly be that part of the DA found in the spinal cord after DOPA administration was formed outside the central nervous system and brought there by the blood and accumulated in the vessel lumen or in the vasomotor nerves. This alternative does not seem very likely, however, as the pia membrane always was carefully removed and it contains most of the large vessels and the sympathetic nerves (Carlsson *et al.* 1964). It is more probable that the difference is due to the DOPA decarboxylase in the capillary walls of the central nervous system [illegible]

importance in the enzyme assays carried out *in vivo* than *in vitro* since all the L-DOPA in this case must first pass through the capillary walls before reaching the decarboxylase containing axons.

The finding that DOPA decarboxylase in the submaxillary glands could be demonstrated only after ligation of their ducts may be due to an enzymatically break down of the decarboxylase in an extract of an intact gland. It has been observed that duct ligation produces a reduction of the gland activity of *i. a.* the protease and amylase (Junqueira 1951). The duct ligation does not diminish the NA content but gives a pronounced concentration of the adrenergic ground plexus due to the atrophy of the parenchyma cells (Andén, Norberg and Olsson 1964). After this operation there is an accumulation of mast cells, and the 5-hydroxytryptophan decarboxylase in them may contribute to the small discrepancy between the minimal levels of the NA content and the DOPA decarboxylase activity obtained after postganglionic sympathectomy. Likewise the mast cells in the rat spleen may be responsible for the similar phenomenon observed in this organ after excision of the celiac ganglion. Nor in the rabbit iris did the DOPA decarboxylase reach the same low level as NA after postganglionic sympathectomy but the agreement was better than in the rat tissues. A great deal of the apparent DOPA decarboxylase activity in the iris, salivary gland and spleen after postganglionic sympathectomy may, however, be due to the non-enzymatic decarboxylation as well as the errors involved in the determination of small amounts of DA. This assumption is strengthened by our finding that after heat denaturation (100° C, 10 min) of an iris extract the value obtained for the DA formation was no less that about 10 per cent of of the control value, *i. e.* of about the same magnitude as after postganglionic sympathectomy.

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The Urinary Excretion and the Tissue Uptake of Noradrenaline after Severe Tissue Depletion of Noradrenaline by α-Methyl-Meta-Tyrosine and Metaraminol

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Abstract

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transmitter but this effect appears to be due to simple displacement rather than interference with transmitter storage and release.

During the last few years a great deal of attention has been paid to α-methyl-m-tyrosine (αMMT) on account of its ability to produce a severe depletion of noradrenaline (NA) from central and peripheral neurons. The active agent is in fact the metabolite metaraminol (α-methyl-β-hydroxy-m-tyramine). Also after injection of this drug there is a drop of the NA in sympathetically innervated organs (for references, see Carlsson 1964 a, Andén 1964). The missing NA is almost stoichiometrically displaced by metaraminol both after administration of this substance and of αMMT (Carlsson and Lindqvist 1962, Carlsson 1964 a, Andén 1964, Udenfriend and Zaltzman-Nirenberg 1964, Shore and Alpers 1964). In an earlier study we succeeded in getting displacement of about 95 per cent of the NA in sympathetically innervated organs of rats and cats. Not even after this intense treatment was it possible to detect impairment of the sympathetic nerve function (Andén and Magnusson 1964). It is to be noted that (—)-metaraminol

is about 3 times weaker in biological activity than (—) noradrenaline This finding has prompted investigations into other aspects of the NA metabolism than the tissue levels after such treatment In this paper results are reported from rat experiments on the NA excretion in the urine and the tissue uptake of labelled NA from the blood after severe displacement of the tissue bound NA by metaraminol

Material and methods

collection The urines from the 3 rats were pooled The tubes were washed with 3 ml N perchloric acid which were added to the urine specimen

The urine specimen was divided into two equal aliquots They were treated in parallel

contents of the heart, spleen and femoral muscles were estimated spectrophotofluorometrically after ion exchange chromatography and oxidation as described by Bertler Carlsson and Rosengren (1958)

The uptake by the heart and femoral muscles of labelled NA from the blood was studied in rats non pretreated as well as rats depleted of NA as described above In both cases (+) NA ^{3}H in a dose of 1 0 μg/kg b w was injected into a tail vein The (±) NA 7 ^{3}H HCl with a specific activity of 5 8 C/mmole was obtained from New England Nuclear Corporation

scintillation counter after ion exchange chromatography (Carlsson and Waldeck 1963)

TABLE I The concentrations of noradrenaline (µg/g, mean ± S E) in different tissues of rats treated with α methyl m tyrosine plus metaraminol

	Heart	Spleen	Femoral muscles
1 No treatment	0 74±0 051 (n=8)	0 42±0 044 (n=5)	0 097±0 0021 (n=4)
2 4 hrs after metaraminol	0 06±0 015 (n=3)	0 03±0 009 (n=4)	0 005±0 0023 (n=4)
3 11 hrs after metaraminol without urine collection	0 08±0 013 (n=3)	0 02±0 003 (n=3)	0 005±0 0025 (n=3)
4 11 hrs after metaraminol with urine collection 4 hrs 11 hrs	0 18±0 014 (n=8)	0 18±0 010 (n=8)	0 012±0 0027 (n=3)
	4-1 p<0 001 4-3 p<0 01	4 1 p<0 001 4-3 p<0 001	4-1 p<0 001 4 3 0 1<p<0 2

TABLE II The effect of combined treatment with α methyl m tyrosine and metaraminol on the urinary excretion of noradrenaline (NA) and adrenaline (A) in adrenal de medullated rats

	NA in µg/kg hr		A in µg/kg hr	
	Control period	Treatment period	Control period	Treatment period
Group 1	0 46	0 33	0 10	0 12
Group 2	0 46	0 44	0 05	0 06
Group 3	0 44	0 70	0 07	0 03
Group 4	0 50	0 62	0 00	0 02
Group 5	0 67	0 49	0 00	0 09
Mean±S E	0 51 ±0 042	0 52±0 065	0 04±0 019	0 06±0 019

Results

The tissue levels of NA After treatment with αMMT (400mg/kg i p on each of the two preceding days) plus metaraminol (0 2 mg/kg i v) there was a considerable reduction of the NA concentrations in the sympathetically innervated organs of rats (Table I) The heart, spleen and femoral muscles were depleted of 90—95 per cent of their original NA content 4 hrs after the administration of the metaraminol The NA levels did not seem to change during the following 7 hrs in the animals not restrained and fixed Unexpectedly, there was a rise of the NA during the same time in the rats from which the urines were collected This phenomenon was apparently most significant in the spleen where the NA concentration increased from about 5 to about 40 per cent of normal

TABLE III The concentrations of tritiated noradrenaline (ng/g mean ± S.E.) in the heart and femoral muscles of control rats and rats treated with α-methyl-m-tyrosine plus metaraminol. The rats were injected with tritiated noradrenaline (1.0 μg/kg i.v.) 30 min before sacrifice

	Heart	Femoral muscles
Controls	3.6 ± 0.22 (n = 5)	0.118 ± 0.0099 (n = 5)
Treated	1.5 ± 0.08 (n = 6)	0.041 ± 0.0083 (n = 6)
	$p < 0.001$	$p < 0.001$

The urinary excretion of NA. The values of the NA excretion in the urine obtained before and after the tissue depletion of NA are presented in Table II. In the control experiments before the displacement of the tissue bound NA by metaraminol the adrenal demedullated rats were found to excrete NA to the urine on the average 0.51 μg/hr and kg b.w. The A excretion was 0.04 μg/hr and kg b.w., *i.e.* the adrenal demedullation appeared successful. (In a few preliminary experiments it was observed that without adrenal demedullation the A and NA excretions were about equal and that this operation changed the NA excretion little.) At least one week after the collection of the control urine the same groups of three rats were treated with αMMT (400 mg/kg i.p. on each of the 2 days preceding the urine collection) and metaraminol (0.2 mg/kg i.v. 4 hrs before the start of the urine collection). Between 4 and 11 hrs after the administration of the metaraminol the rats excreted on the average 0.52 μg NA and 0.06 μg A per hr and kg b.w.

The tissue uptake of NA-³H. After a severe displacement of the tissue bound NA with metaraminol there was a significant reduction of the tissue uptake of labelled NA from the blood (Table III). In the control rats the heart and femoral muscles contained 3.6 and 0.118 ng/g (ng = 10^{-9} g) NA-³H respectively 30 min after an i.v. injection of 1.0 μg per kg b.w. NA-³H. The same dose was given i.v. 3 hrs and 45 min after metaraminol (0.2 mg/kg i.v.) to rats pretreated with αMMT (400 mg/kg i.p. on each of the 2 preceding days). At sacrifice 30 min later there was 1.5 and 0.041 ng/g NA-³H in the heart and femoral muscles respectively, *i.e.* 42 and 35 per cent of the control values.

Discussion

Like reserpine αMMT plus metaraminol can produce an almost complete depletion of the tissue NA. After reserpine treatment stimulation of the sympathetic nerves fails to evoke the normal functional response (Carlsson *et al.* 1957, Muscholl and Vogt 1958). This reserpine effect is probably due to an inhibited release of NA from the sympathetic nerve endings since the output of NA to the blood obtained in untreated animals at intense ganglionic stimulation by carbacholine is almost completely blocked after reserpine treatment (Bertler *et al.* 1958). Reserpine also causes a considerable drop in the urinary NA of rabbits (Carlsson *et al.* 1957), rats (Leduc 1961, Johnson 1963) and humans (Gaddum, Krivoy and Laverty 1958, Carlsson, Boje Rasmussen and

Kristjansen 1959) These urinary findings support the view that the NA in th mainly originates in postganglionic sympathe
the a
after
there ... the nerve endings after this
treat ... rpretation of the results obtained in the present study gives an explanation of our previous observation that this treatment does not impair the sympathetic nerve function although at least 90 per cent of the NA in the tissues is displaced by the biologically about 3 times less active metaraminol (Andén and Magnusson 1964)

The urinary excretion of NA was 2—7 times higher in the present investigation than in earlier ones where the urine was collected from rats placed in metabolic cages (Schapiro 1958, Crawford and Law 1958, Hökfelt and Bygdeman 1961, Perman 1961, Leduc 1961, Gunne 1963, Johnson 1963) The discrepan
fact that the urine
procedure we avoid
the collection bottle ... There are however, also other explanations of the high NA excretion It has been observed that a subcutaneous injection of a small volume of saline to rats increases the urinary excretion of NA (Crawford and Law 1958) an effect probably not caused by increased diuresis (Euler 1956, p 167), and in all our experiments about 10 ml
restrained and fixed
Increased musc
to the urine (Eu ... be a disadvantage that the impulse flow of the sympathetic nerves was high in our urine experiments
know if the NA release from the ner
cold stress, when the sympat
urine after reserpine treatment ... atively to untreated controls than at room temperature (Johnson 1963)

It is not unlikely that an increased impulse flow of the sympathetic nerves also was the cause of the significant rise of the NA levels in the animals used for the urine collection A high sympathetic tone should produce an increased release of the metaraminol from the store and simultaneously, an increased synthesis of NA (Euler and Hellner Björkman 1955) It is likely that the release of metaraminol from the nerve endings was of an order of magnitude much smaller than that of NA Part of the newly synthesized NA was probably taken up in the store, replacing the lost metaraminol The NA levels did not rise very rapidly however and were significantly depressed compared with the untreated controls also at the end of the urine collection, i e the urinary output of NA appeared unchanged despite severely depleted tissue stores of NA during the whole period

Reserpine blocks almost completely the uptake of NA from the blood by the sympathetic nerves of peripheral organs (Muscholl 1960 Hertting Axelrod and Whitby 1961) There is a certain temporal correlation between NA uptake and adrenergic nerve function after reserpine treatment the uptake is almost completely inhibited as long as the adrenergic transmission is blocked
uptake
... much more slowly than the uptake mechan

ism It is noteworthy that in the present investigation the same discrepancy between NA uptake and NA concentration existed after treatment with αMMT plus metaraminol Hess *et al* (1961) reported that αMMT to guinea pigs reduced the uptake of labelled NA by the heart also by some 60 per cent but in their experiments the NA drop was only about 70 per cent It seems, thus, as the adrenergic transmission is unimpaired at a NA uptake about 40 per cent of the normal, since this value was obtained both after treatment with αMMT plus metaraminol and at the recovery from the sympathetic blockade produced by reserpine As mentioned above, at both instances the sympathetic nerves function apparently unimpaired although the sympathetic innervated organs are depleted of at least 90 per cent of their NA From these data it appears that only a small fraction of the NA in store is immediately necessary for nerve function Hillarp (1960) was the first to discover the non homogeneous nature of the adrenergic store by showing that the catecholamines in the adrenal medullary granules occur in two pools one large stable fraction bound to an equivalent amount of adenosine phosphates and one small labile fraction bound in an unknown manner Monoamines seem to be primarily incorporated into the small fraction both in adrenal medullary and nerve granules and it is probable that the amine release takes place to a normal extent only when this mechanism is intact (for references, see Carlsson 1964 b) There are several possibilities of the difference between the uptake of NA ^{3}H in the animals untreated and those given αMMT plus metaraminol or those in the recovery phase after reserpinization For example in the non pretreated animals approximately 60 per cent of the NA ^{3}H may be accumulated in the large functionally rather inert fraction and the incorporation into this fraction may be blocked for a long time after treatment with αMMT plus metaraminol or with reserpine Anyhow, even after the severe depletion of the tissue NA by αMMT plus metaraminol the sympathetic nerves can incorporate a considerable amount of NA-^{3}H and this ability may explain why the NA release and thus the adrenergic transmission is apparently unimpaired

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Properties of Afferent Connections to the Rostral Spinocerebellar Tract in the Cat

By

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Abstract

Oscarsson, O. and N. Uddenberg. *Properties of afferent connections to the rostral spinocerebellar tract in the cat.* Acta physiol. scand. 1965. 64. 143—153. — Properties of the rostral spinocerebellar tract (RSCT) have been studied and compared with those of its functional hindlimb equivalent, the ventral spinocerebellar tract (VSCT). Both RSCT and VSCT are monosynaptically activated from high threshold Group I muscle afferents and polysynaptically influenced from the flexor reflex afferents. Excitation from Group I afferents is mono-, di-, and polysynaptic in the RSCT, but exclusively monosynaptic in the VSCT. The monosynaptic linkage has different properties in the two tracts. The effects from the flexor reflex afferents are predominantly excitatory in the RSCT and predominantly inhibitory in the VSCT. The significance of similarities and differences in the organization of the two tracts is discussed.

The rostral spinocerebellar tract (RSCT) is activated from ipsilateral forelimb nerves. It is anatomically distinct from the dorsal spinocerebellar tract (DSCT) in arising from cell bodies rostral to Clarke's column and in occupying a relatively ventral position in the cord, and from the ventral spinocerebellar tract (VSCT) in being uncrossed. In addition, the RSCT differs from these tracts in reaching the cerebellum through the restiform body as well as the brachium conjunctivum (Oscarsson and Uddenberg 1964).

The DSCT and VSCT have no components related to the forelimbs and front part of the body (Holmqvist, Oscarsson and Uddenberg 1963b). The RSCT is, however, a functional equivalent of the VSCT. This is demonstrated by the general similarity in the organization of afferent connections and the similar mode of termination in the cerebellar cortex (Oscarsson 1964b, Oscarsson and Uddenberg 1964). Both RSCT and VSCT are monosynaptically activated from high threshold Group I muscle afferents and polysynaptically influenced from the flexor reflex afferents (FRA,

This paper describes the properties of the synaptic linkage between Group I afferents and RSCT neurones The characteristics of the FRA connections to these neurones will also be reported. The significance of similarities and differences in the organization of the RSCT and VSCT will be discussed

Methods

The experiments were done on cats The body temperature was kept between 36 and 38.5° C. The blood pressure was continuously recorded and prevented from falling below 80 to 90 mm Hg Artificial ventilation was used in most experiments.

a Mass discharge recording The animals were usually under pentobarbitone anaesthesia In the left forelimb the following nerves were dissected and mounted for stimulation the muscle component of the suprascapular nerve (SSC), the axillar nerve (Ax) the nerve to the long head of the triceps (LHT) the biceps nerve (B), the deep radial nerve (DR), the superficial radial nerve (SR) the median nerve (Med), and the ulnar nerve (Uln) The radial nerve

listed above The right hamstring nerve was prepared for stimulation in order to permit

procedure together with bilateral pneumothorax, prevented respiratory and circulatory movements allowing stable recording with capillary microelectrodes from fibres in the lateral funiculus The afferent volleys were recorded triphasically from the dorsal funiculus at the same segmental level (C3)

Ia and "*Ib afferents* are used synonymously with large muscle spindle and tendon organ afferents *Ipsilateral* and "*contralateral* refer to the side of the ascending axons *Stimulus strength* is expressed in multiples of the strength necessary for evoking a barely visible ingoing volley

Results

A comparison between the RSCT and VSCT is of considerable interest as these two tracts related to the forelimbs and hindlimbs respectively, can be regarded as functional equivalents Most of the experiments were designed to permit recording from both tracts in the same animal Identification of the tracts was based on the finding that ascending spinal neurones activated monosynaptically from Group I muscle ... SCT

... tion

... tail

... and

Oscarsson 1962) and our observations on this tract are in agreement with those already reported.



Fig 1 Re... reco... affer... segn... co dates

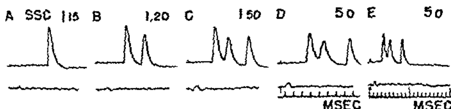
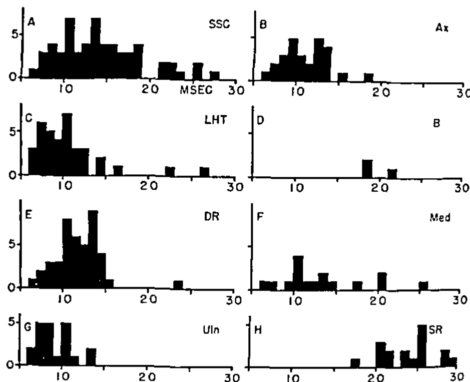


Fig 2 Recording from RSCT unit (upper trace ... the suprascapular ...

1 Connections with Group I afferents

a Synaptic linkage The response evoked in RSCT neurones by a volley in Group I afferents has been studied by recording from more than 110 units in 15 expts The number of connections investigated is larger as most of the RSCT neurones received Group I excitation from more than one nerve The response was usually a single spike with a short latency indicating a monosynaptic linkage For example, in Fig 1 stimulation of the deep radial nerve evoked an impulse with a latency of 1 0 msec as measured relative to the afferent volley recorded at the same segmental level This value corresponds approximately to the "synaptic delay" as the conduction velocity is about the same in the primary afferents ascending in the dorsal funiculus and in the tract fibres (Rexed and Ström 1952 Oscarsson and Uddenberg 1964

Observations made on some RSCT units indicate that di and polysynaptic excitation from Group I afferents also occurs In occasional units a spike appeared after a latency of more than 2 msec at a low strength This latency decreased abruptly to 1 msec or less at higher strengths of stimulation A unit with this behaviour was illustrated in a previous paper (Oscarsson and Uddenberg 1964 Fig 1 In other units the latency was long also on stimulation at maximal Group I strength The unit shown in Fig 2 was activated by volleys in the suprascapular nerve At a low strength producing only a small afferent volley A an action potential appeared after a latency of 4 0 msec With a large Group I volley the latency decreased to 2 3 msec (B, C Units of this



type were usually activated by Group I afferents in other nerves after a latency proving a monosynaptic linkage and can hence be identified as RSCT neurones

Action potentials evoked after a latency indicating a di- or polysynaptic linkage were often observed on stimulation of the suprascapular nerve but occurred also on stimulation of Group I afferents in the other nerves tested, as shown by the histograms in Fig 3, A—G It should be noted that the discharge evoked by a volley in the biceps nerve, in each of the 3 cases observed, had a latency indicating intercalation of at least one interneurone (Fig 3, D) Histograms A—E sample spikes evoked by Group I volleys that were less than 70 per cent maximal This would exclude any appreciable contribution from Group II afferents in most cases Histograms F and G, relating to the mixed median and ulnar nerves, were obtained by sampling responses evoked at stimulus strengths below 1 3 times threshold which would exclude contribution from cutaneous afferents and Group II muscle afferents in the majority of the cases

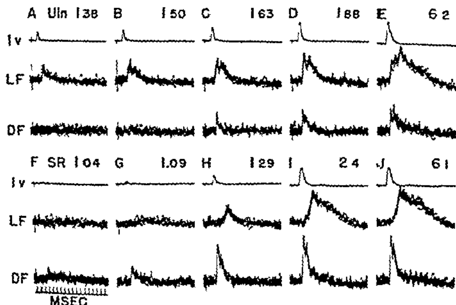


Fig 4 Experiment to demonstrate threshold separation between Group I afferents and cutaneous afferents in the ulnar nerve The traces show from above downwards afferent volley in the dorsal funiculus (I v) mass discharge in tracts ascending in the intermediate one third of the lateral funiculus (lateral fascicle LF) and mass discharge in tracts ascending in the dorsal one third of the lateral funiculus (dorsal fascicle DF) The dorsal funiculus and the dorsal and ventral fascicles were dissected and mounted for recording at the C3 level A—E stimulation of the ulnar nerve (Uln) and F—J stimulation of the superficial radial nerve (SR) at indicated strengths

The threshold separation between Group I afferents and cutaneous afferents in the ulnar nerve is illustrated in Fig 4 The mass discharge evoked in a lateral fascicle containing the RSCT was compared with the mass discharge evoked in a dorsal fascicle containing the dorsomedial cutaneous tract which presumably is identical with the spinocervical tract (*Holmqvist et al* 1963 b) On stimulation of the ulnar nerve the RSCT discharge was appreciable at 1 38 (A) and large at 1 50 times threshold (B The discharge in the cutaneous tract appeared at 1 5 times threshold (B) There was little need for spatial summation in the cutaneous tract as illustrated by records F—J (lower traces obtained on stimulation of the superficial radial nerve Hence it can be concluded that few cutaneous afferents were included in the volley from the ulnar nerve before the stimulus strength had been raised above 1 5 times threshold Similar observations were made in other experiments on stimulation of the median and ulnar nerves and show that few if any cutaneous afferents were activated by stimuli below 1 3 times threshold

The RSCT unit shown in Fig 2 responded with a repetitive discharge to stimulation of Group I afferents in the suprascapular nerve B C) The low threshold argues against the possibility that the later impulses were produced by Group II afferents and so does the absence of further spikes at higher strengths with activation of many Group II afferents D E A repetitive discharge produced by a Group I volley was relatively often observed on stimulation of the suprascapular nerve The initial spike had either a short monosynaptic or long latency Repetitive activity

RSCT VSCT

MSEC

MSEC

Fig 5 Configuration of mass discharge in RSCT and VSCT as recorded from the lateral funiculus dissected in the third cervical segment The discharges were evoked by slightly submaximal Group I volleys from the ipsilateral deep radial nerve (RSCT) and the contralateral hamstring nerve (VSCT) The upper and lower records were obtained simultaneously at different speeds

evoked by Group I volleys in the other nerves (*cf* Oscarsson 1965a Table I) Presumably the repetitive discharge was due to prolonged, asynchronous bombardment from interneurones

The records in Fig 5 show, on a fast (upper traces) and slow time base, the mass discharges in the RSCT and VSCT evoked by volleys in Group I afferents and recorded from the lateral funiculus dissected in the upper cervical region The difference in configuration of the discharges is conspicuous The long duration of the RSCT discharge is due to the late activity evoked in the RSCT units and described above The duration was long already when the discharge first appeared on stimulation of muscle nerves at 1 2—1 4 times threshold strength and did not change markedly with additional activation of more Group I afferents (Holmqvist *et al* 1963b Fig 2, Q—T) This is in agreement with the observations made on single fibres and suggests that the early and late spikes in the RSCT units were evoked by the same category of high threshold Group I afferents The short duration of the mass discharge in the VSCT (Fig 5) is explained by the brief EPSPs (excitatory post-synaptic potentials) which are evoked in the tract neurones through their exclusively monosynaptic connections with Group I afferents (Eccles *et al* 1961a)

The front of the discharge in the RSCT was less steep than that in the VSCT (Fig 5) The reason for this difference is obscure The front is formed by the monosynaptically elicited spikes in the individual neurones The Group I volley from forelimb nerves (Holmqvist *et al* 1963b Fig 2, A—H) is as synchronous as that from hindlimb nerves and it seems unlikely that unequal conduction velocities of the RSCT fibres would introduce any significant temporal scatter considering the short conduction distance in the RSCT (about 4 5 cm) Perhaps the primary afferent collaterals leading to the RSCT neurones have widely different conduction velocities and therefore activate these neurones after varying delays

b Synaptic properties It has previously been shown that the properties of the synaptic linkage between Group I afferents and VSCT neurones in some respects differ from those of the synaptic linkage between Group I afferents and DSCT neurones (Eccles *et al* 1961 a, Eccles Oscarsson and Willis 1961 b) The observations described below demonstrate differences in the properties of the monosynaptic linkage to RSCT and VSCT neurones respectively

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Fig 6
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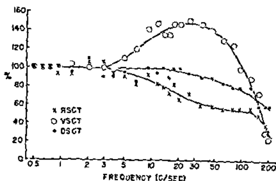


Fig 6 Effect of frequency on amplitude of mass discharge in the RSCT, VSCT, and DSCT. The amplitude as percentage of the slowest frequency response is plotted against frequency of stimulation The mass discharges were recorded from the lateral funiculus dissected and mounted for stimulation at the C3 level The discharge in the RSCT was evoked by combined stimulation of the ipsilateral deep radial nerve and the ipsilateral nerve to the long head of triceps, the discharge in the VSCT by combined stimulation of the contralateral hamstring and triceps surae nerves, and the discharge in the DSCT by combined stimulation of the corresponding ipsilateral nerves

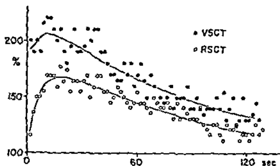


Fig 7 Post tetanic potentiation of mass discharge in RSCT and VSCT Stimulating and recording arrangement as in Fig 6 Ordinate amplitude of mass discharge as percentage of value obtained before the tetanus Abscissa time after tetanus (duration, one minute, frequency 300 stimuli per second) The testing discharges were elicited at a frequency of 0 55 per second

against stimulation frequency The measurements were made after a steady value had been reached after about 10 stimuli at the low frequencies and after a much larger number of stimuli at the high frequencies The mass discharge in the VSCT increased at frequencies above 3—5 per sec, became maximal at 10—50 per sec and decreased below the control value at frequencies above 100 per sec The increase of the VSCT discharge on repetitive stimulation is due to a corresponding increase in the size of the Ib EPSPs in VSCT neurones (Eccles *et al* 1961 a) On the other hand, the RSCT discharge decreased in amplitude at frequencies above 3 per sec This suggests that the EPSPs in RSCT neurones decrease in size on repetitive stimulation just as the Ia EPSPs in limb motoneurones (Curtis and Eccles 1960) and the Ia and Ib EPSPs in DSCT neurones (Eccles *et al* 1961b) The DSCT discharge did not decrease until the rate of the stimulation was increased above 15—20 per sec, whereas the EPSPs in DSCT neurones start to decrease in size already at frequencies above 1 per sec (Eccles *et al* 1961b) Presumably the resistance of the DSCT discharge at higher frequencies was due to a large safety margin of the synaptic linkage (cf Oscarsson 1965b)

Complementary information was obtained by recording from single units Most RSCT and VSCT neurones followed presynaptic stimulation rates of several hundreds per sec initially After a period of stimulation (several seconds) the highest frequency

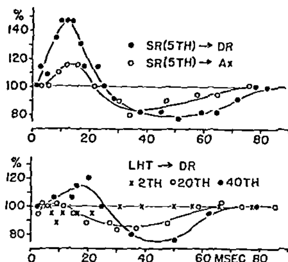


Fig 8 Effects on RSCT mass discharge produced by conditioning volleys in cutaneous and muscle afferents Upper graph the conditioning volley was elicited in the superficial radial nerve (SR) and the testing volley in the deep radial (DR) and axillar nerve (Ax) respectively Lower graph the conditioning volley was elicited in the nerve to the long head of triceps (LHT) and the testing volley in the deep radial nerve The conditioning volleys were elicited at the stimulation strengths indicated on the graphs The amplitude of the conditioned discharge in per cent of the unconditioned one is plotted against volley interval in msec

that was followed faithfully had decreased to between 100 and 200 per sec in most RSCT and VSCT neurones

Previous investigations have demonstrated that the post-tetanic potentiation of the mass discharge in the VSCT is much larger than in the DSCT, though smaller than the potentiation of the monosynaptic reflex (Oscarsson 1957) The potentiation following a prolonged tetanus is preceded by a depression which has a shorter duration in the VSCT and DSCT than in the monosynaptic reflex (Lloyd 1949, Oscarsson 1957) It has now been found that the post-tetanic potentiation in the RSCT resembles that in the other two spinocerebellar tracts in having an initial depression of short duration (Fig 7) This depression was somewhat more conspicuous in the RSCT than in the VSCT (Fig 7) In the three experiments performed the potentiation was less in the RSCT than in the VSCT (Fig 7), though it was larger than the potentiation usually found in the DSCT (Holmqvist, Lundberg and Oscarsson 1956, Oscarsson 1957, McIntyre and Mark 1960)

2 Connections with FRA

The effects produced in RSCT neurones by volleys in cutaneous and high threshold muscle afferents were studied on mass discharge and unit recording A conditioning cutaneous volley always facilitated the RSCT mass discharge, irrespective of whether the testing discharge was evoked from a muscle nerve innervating structures close to, or distant from those innervated by the cutaneous nerve (Fig 8, upper graph) A slight depression usually followed the facilitation

Volleys in high threshold muscle afferents were less effective There was usually a weak facilitation followed by some depression, but sometimes only a weak (less than 15 per cent) facilitation or inhibition In the example shown in Fig 8 (lower graph) a conditioning volley in Group I afferents (crosses) produced no appreciable effect whereas additional activation of Group II and III muscle afferents produced some inhibition (open circles) and at higher strengths of stimulation also some initial facilitation (filled circles)

A volley in FRA afferents often elicited a discharge in the RSCT neurones For example, in Fig 1 stimulation of high threshold (Group II and III) muscle afferents (D—F) and cutaneous afferents (G) produced repetitive activity The latency of the first impulse elicited from the skin nerve and measured relative to the afferent volley recorded at the same segmental level, varied from 1 7 to many msec The shortest latencies are sampled in the histogram of Fig 3, H The first spike was usually evoked at a low stimulus strength and the latencies indicate that the transmission was di or polysynaptic The minimal latency of the spikes evoked from high threshold muscle afferents was 1 4 msec longer than the shortest latency of the spikes evoked from Group I afferents This latency is too long to be explained alone by the slower conduction velocity in Group II afferents It suggests that the synaptic linkage was di- or polysynaptic

Discussion

The RSCT is a forelimb equivalent of the VSCT This is demonstrated by its mode of termination in the cerebellum which is similar to the termination of the VSCT and characteristically different from the termination of the DSCT and the cuneocerebellar tract (Oscarsson and Uddenberg 1964) It is also demonstrated by the general similarity in the organization of the afferent connections to the RSCT and VSCT Both tracts are monosynaptically activated from high threshold muscle afferents which can be identified as tendon organ afferents (Oscarsson 1956, 1957 1960 1965a Eccles *et al* 1961a Holmqvist *et al* 1963b) and polysynaptically influenced from the FRA. Furthermore the convergence of Group I excitation to individual neurones is similar in the RSCT and VSCT In both tracts the convergence is extensive each neurone being usually activated from synergic muscle groups at several joints, sometimes even from one muscle group at each of the main joints of the limb (Oscarsson 1957 1965a Eccles *et al* 1961a)

However there are also conspicuous differences in the organization of the two tracts Group I afferents activate RSCT neurones not only through a monosynaptic linkage but also through di and polysynaptic connections. On the other hand the linkage between Group I afferents and VSCT neurones is exclusively monosynaptic (Eccles *et al* 1961a) The monosynaptic linkage has different properties in the two tracts in the RSCT transmission is depressed on repetitive stimulation at frequencies between 5 and 100 per sec whereas it is facilitated in the VSCT This is presumably due to corresponding changes in the size of the EPSPs that are elicited in the tract neurones The time course and magnitude of the post tetanic potentiation were also different in the RSCT and VSCT The significance of the differences in synaptic properties is unknown they presumably represent adaptations to special functions Differences of the same order have been observed in the properties of the synaptic connections of other functionally related pathways For example the Ia EPSPs in limb motoneurones decrease in size on repetitive stimulation whereas they increase in respiratory motoneurones of the thoracic cord Curtis and Eccles 1960 Sears 1964

The FRA effects are predominantly excitatory in the RSCT and predominantly inhibitory in the VSCT This difference between the two tracts may be of little general significance as it is known that some VSCT neurones in the cat receive predominantly excitation from the FRA Oscarsson 1957 and that excitation is the dominant effect in the VSCT of the dog Oscarsson Rosén and Uddenberg 1964

In addition to these functional distinctions between the RSCT and VSCT *there is* one major anatomical difference [illegible] and the VSCT crossed at the segmental level [illegible] when related to the recent disclosure that u[illegible] tracts differ in fundamental respects (Oscarsson 1956 1964a, Magni and Oscarsson 1962, Holmqvist and Oscarsson 1963, Szentagothai 1964) The uncrossed tracts ascend in the dorsal part of the lateral funiculus and originate from cell bodies in the dorsal or dorsomedial part of the spinal grey matter The crossed tracts ascend in the ventral part of the lateral funiculus and in the ventral funiculus and originate from cell bodies in the ventral or ventrolateral part of the grey matter Furthermore, the uncrossed tracts have ipsilateral receptive fields of polysynaptic actions, whereas the crossed tracts have bilateral fields In accordance with the latter "rule" it has been found that the polysynaptic effects from the FRA are ipsilateral in the RSCT and bilateral in the VSCT (Oscarsson 1957, 1965a)

The fact that the RSCT belongs to the uncrossed tracts and the VSCT, to the crossed tracts suggests that they have arisen independently from different embryological anlages by parallel evolution of similar patterns of organization The two tracts would be analougous rather than serially homologous structures The reason why the hind limb and forelimb tracts have developed as separate anatomical entities is unknown A similar situation is encountered in connection with the DSCT and the cuneocerebellar tract These two pathways relate to the hindlimbs and forelimbs respectively and contain largely equivalent channels for proprioceptive and exteroceptive information (Holmqvist, Oscarsson and Rosén 1963a) On the other hand, there is no evidence for anatomical differences between the forelimb and hindlimb components of the major pathways to the cerebrum, the dorsal funiculus-medial lemniscus system and the spinocervical tract (Kruger Siminoff and Witkovsky 1961, Andersson 1962 Oswaldo-Cruz and Kidd 1964), though it has recently been shown that the former system contains a forelimb but not a hindlimb channel for information from large muscle spindle afferents (Oscarsson and Rosén 1963)

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From the Institute of Physiology, University of Lund, Sweden

Integrative Organization of the Rostral Spinocerebellar Tract in the Cat

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Abstract

Oscarsson O *Integrative organization of the rostral spinocerebellar tract in the cat* Acta physiol scand. 1965 *64* 154—166 — The convergence of synaptic actions from Group I muscle afferents and flexor reflex afferents (FRA) to rostral spinocerebellar tract (RSCT) neurones, and the effects produced in these neurones by natural stimulation of receptors have been investigated The monosynaptic excitation from Group I afferents was related mainly or exclusively to tendon organ afferents The convergence of Group I excitation to individual tract neurones was usually extensive there was often excitation from one muscle group at each of the shoulder elbow and wrist and finger joints The RSCT neurones received polysynaptic excitation from the FRA of large receptive fields in the ipsilateral forelimb These fields had no obvious relation to the Group I receptive fields The effects evoked by stimulation of cutaneous receptors were weak The FRA effects from muscle were often strong on maintained stretch there was only moderate adaptation and cessation of stretch was followed by an afterdischarge The organization in the RSCT and VSCT (ventral spinocerebellar tract) is compared and the information forwarded by the two tracts is discussed — Additional observations demonstrated that VSCT neurones were influenced from the FRA not only in hindlimb but also in forelimb nerves. There is a discussion of the significance of this finding for a hypothesis concerning the informative value of the FRA connections to ascending tracts.

The course and termination of the rostral spinocerebellar tract (RSCT and the general organization of the afferent connections to this tract were described in previous papers (Oscarsson and Uddenberg 1964 1965 The RSCT is activated monosynaptically from high threshold Group I afferents and polysynaptically from the flexor reflex afferents FRA The RSCT is a functional equivalent of the ventral spinocerebellar tract VSCT as demonstrated by the similar mode of termination in the cerebellar cortex and the similar organization of afferent connections (Oscarsson 1964b Oscarsson and Uddenberg 1964 The receptive fields of the RSCT neurones are described in this paper together with observations on natural stimulation of receptors The findings permit a further comparison of corresponding features in the RSCT and

VSCT The experiments have been designed to permit recording from both these tracts in the same animal and some new observations on the VSCT have been added to those described previously (Oscarsson 1956, 1957, 1960, Eccles, Hubbard and Oscarsson 1961, Lundberg and Oscarsson 1962)

Some of the findings have been reported in brief (Oscarsson 1964b)

Methods

The technique and the preparations have been described in previous papers (Oscarsson and

limbs

Some experiments were designed to study effects on natural stimulation of muscle receptors The ipsilateral forelimb was denervated except for the deep radial and median nerves which were mounted on stimulating electrodes and left in connection with the periphery The tendons of the following muscles were prepared for pulling by hand or loading extensor carpi radialis extensor digitorum communis extensor digitorum lateralis, extensor carpi ulnaris flexor profundus digitorum, palmaris longus and flexor carpi radialis Gallamine was given in order to protect the nerves from damage due to muscle contractions

Ipsilateral" and "*contralateral*" refer to the side of the ascending axons *Stimulus strength* is given in multiples of the strength necessary for evoking a barely visible ingoing volley

Results

1 *Patterns of convergence*

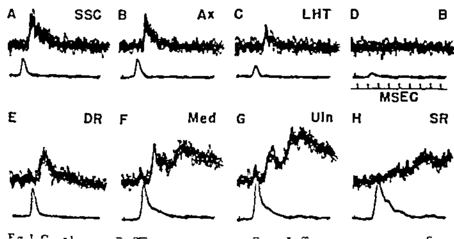
a Group I afferents The contribution from Group I afferents in various nerves to the activity in the RSCT was studied by recording the mass discharge in the tract In Fig 1 the intermediate one third of the lateral funiculus was dissected for recording in the upper cervical cord This part of the funiculus contains the RSCT but not the dorsomedial cutaneous tract with its prominent monosynaptic discharge evoked from cutaneous afferents (Holmqvist Oscarsson and Uddenberg 1963b) The RSCT discharge evoked by a maximal Group I volley in 5 muscle nerves is shown by records A—E Stimulation of the suprascapular, axillar and deep radial nerves produced large discharges, the triceps (LHT) nerve a small discharge and the biceps nerve a hardly visible discharge The mixed median and ulnar nerves were stimulated at 2 5 times threshold strength which would activate cutaneous afferents and Group II muscle afferents in addition to the Group I afferents Oscarsson and Uddenberg 1965 Fig 4) Stimulation of the skin nerve only evoked a small early response (H in this part of the funiculus It can be concluded that the initial monosynaptic component of the mass discharges evoked from

TABLE I Convergence of excitatory action to 40 RSCT units in 3 experiments (I—III) Abbreviations of nerves are explained in Fig 1 Excitatory action (discharge evoked by

Exp	Nr	SSC	Ax	LHT	B[1]	DR	Mrd	Uln	SR	Conv
I	1	+ r F	+ F	+	F	+ F	F	+ F	F	5F
	2	+	+	+		F	+ F	+ F		5F
	3	+		+			+	+		4
	4	(+) r	+ r		F	+		+		3F
	5	(+)	+			+		+		3
	6	F	+ F					+		3F
	7		F	+ F		+ F	F	+ F	F	3F
	8		+	+				+		3
	9	F	+		F	+		+		3F
	10	+ r F	+ F				F	F		2F
	11		+ F				+ F			2F
	12	(+) rF		+						1F
	13						+ F			1F
II	14	+		+		+		+		4
	15	+				+ r F	(+) F	+ r F	F	3F
	16	( ) r F	+			+		+		3F
	17	+ F	+ F			F	(+) F	F	F	3F
	18	+ r F	+ F			+	F	F		3F
	19	+ r	+	+						3
	20	+ r F	+ F	F		F	F	F	F	2F
	21						+	+ r	F	2F
	22	r	+							2
	23	r								2
	24	+ r								2
	25						(+) F	+ F	F	1F
	26						(+)	+ r F		1F
	27		+ r			F	F			1F
	28			+						1
	29	+								1
III	30	+ F	+ F	F	F	+ F	F	F	F	3F
	31	F	+ F	F	F	+ F	F	F	F	3F
	32	+ F	+ F	F	F	+	F	F	F	3F
	33	F	+ F			+	F			3F
	34			F		F	F	+ F	F	3F
	35	F	F	F	F	F	F	F	F	2F
	36	F	+ F	F						2F
	37	F	+ F							2F
	38	F	F	F	F	+ F	F	+ F	F	1F
	39	F	F			F	F	F	F	1F
	40	F					F	+ F		1F

[1] Not stimulated in exp II

single volley) is indicated as follows —, monosynaptic activation from Group I (+) di or polysynaptic activation from Group I, r, repetitive activation from Group I, F, activation from FRA. Monosynaptic excitation from Group I arbitrarily assumed when the latency of the response was less than 1 5 msec as measured relative to the afferent volley recorded at the same segmental level (cf Oscarsson and Uddenberg 1965) Column to extreme right figure, number of nerves supplying monosynaptic Group I activation F, FRA activation from at least one nerve



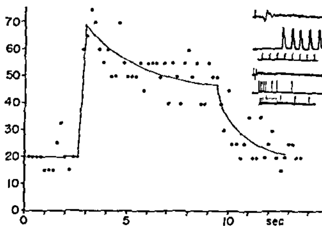
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the mixed nerves (F, G) largely represents activity in the RSCT This and similar experiments showed that physiological extensors (SSC, LHT, Med) as well as flexors (B, DR) contribute Group I excitation to the RSCT The size of the RSCT discharges evoked from the various nerves was roughly proportional to the size of the ingoing Group I volleys

Information about the convergence of monosynaptic excitation to individual RSCT neurones was obtained by recording from single fibres The following discussion is based on observations of 116 units encountered in 15 expts In three of theses 8 ipsilateral nerves could be stimulated and are listed in Table I In the remaining experiments the [illegible]

usually convergence from several nerves and sometimes from as many as five One unit with extensive convergence is illustrated in Fig 2 It was monosynaptically activated from Group I afferents in the suprascapular triceps, deep radial and ulnar nerves

Several of the nerves used for stimulation are compound and innervate muscles of diverse function (cf Reighard and Jennings 1935) This obscures the patterns of con-



typical unit is shown in Fig 3 It was monosynaptically activated from Group I afferents in the triceps, deep radial, and ulnar nerves (C, E, H) and polysynaptically activated from the FRA in the axillar, triceps, deep radial, median, ulnar, and superficial radial nerves There was no obvious correlation between the receptive fields related to Group I afferents and FRA respectively

2 *Natural stimulation of receptors*

a Muscle receptors The effects from receptors in and around muscle were investigated in five experiments The ipsilateral forelimb was denervated except for the deep radial and median nerves which were mounted on stimulating electrodes but left in connection with the periphery The tendons of some of the muscles innervated by these nerves were severed and prepared for pulling or loading (see Methods) RSCT units were identified either by being monosynaptically activated from Group I afferents in the deep radial or median nerve or from Group I afferents in the suprascapular or triceps nerve which were cut and mounted for stimulation

Units activated from Group I afferents but not the FRA in either the deep radial or the median nerve were tested by pulling or loading of the appropriate tendons Activity was usually evoked on pull of one or several tendons, but usually not on pull of all the tendons, suggesting a certain restriction of the receptive field related to a group of synergistic muscles Most units had a resting activity and the response to maintained pull or to loading was an increased frequency which after a short period of moderate adaptation remained at a steady level (Fig 4) On cessation of loading there was an abrupt decrease in the frequency which returned to the resting level either immediately (Fig 4, B), or after a transient depression (A) A quick stretch of the muscle produced no burst of activity as would be expected if muscle spindle afferents contributed to the

excitation The threshold was only roughly determined Usually a load of 50 to 150 g was necessary for producing an appreciable increase in the resting activity

The RSCT is activated from high threshold Group I afferents in ipsilateral forelimb nerves (Holmqvist *et al* 1963b) The high threshold Group I afferents in hindlimb nerves have been identified as tendon organ afferents in the cat (Bradley and Eccles 1953 Eccles Eccles and Lundberg 1957, Laporte and Bessou 1957) and several other mammalian species (Magni and Oscarsson 1962 Oscarsson Rosén and Uddenberg 1964) The results on receptor stimulation described above indicate that this identification holds true also for the high threshold Group I afferents in forelimb nerves of the cat The properties of the receptors conformed to those of tendon organs slow adaptation, high mechanical threshold, and no special sensitivity to quick stretch Furthermore, the results in a recent investigation (Oscarsson and Rosén 1963) suggest that the low threshold Group I afferents in forelimb nerves originate from muscle spindles

Stretch of muscles supplying FRA but not Group I excitation sometimes produced a short lasting discharge which returned to the resting level after a few seconds However, there was usually only moderate adaptation on maintained stretch and cessation of stretch was then followed by an afterdischarge (Fig 5) The receptors responsible for the slowly adapting effects are unkown Group II afferents with flower spray endings might be partly or mainly responsible The afterdischarge was presumably due to a continued activity *in the interneurones on the path from the FRA to the tract* neurones

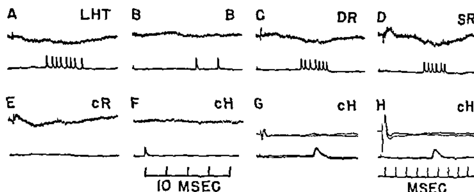
RSCT units which seemed to be little affected by stretch of the forearm muscles were sometimes strongly activated by bending of the wrist and toe joints These effects appeared when the joint was rotated towards extreme extension or flexion but before pain receptors were likely to be activated There was usually only moderate adaptation on maintained bending In some cases the foot was dissected for localization of the receptors There was no effect on pressure against the joint capsules or on movement in the individual small joints but usually strong activation on pressure against deep structures in the foot Presumably the receptors were situated in ligaments and other connective tissue

b Cutaneous receptors A volley in the skin nerve, the superficial radial nerve often produced a discharge in the RSCT units (Fig 3 Table I) Stimulation of cutaneous receptors only occasionally evoked activity in these units This activity was produced by pressure pinching and exceptionally also by touch The effects were obtained from vaguely delimited areas on the distal part of the limb (Fig 3) On maintained stimulation there was only moderate adaptation

In many cases stimulation of the skin nerve evoked a distinct, or even intense discharge in the RSCT unit while stimulation of cutaneous receptors was ineffective This discrepancy between the effects evoked by a synchronous volley and the asynchronous barrage of impulses on natural stimulation was in some cases observed also in connection with stimulation of high threshold muscle afferents and calls for caution when interpreting receptive fields on the basis of observations made on nerve stimulation

Inhibitory effects were observed in a few units on cutaneous stimulation and on pulling of tendons These effects were weak and of doubtful significance

c Limitation of receptive fields Supramaximal stimulation of the contralateral radial nerve was tried with many RSCT units. No discharge was elicited in the vast majority of the units In the remaining units one or two spikes were evoked after a long latency



nerve.

Intense stimulation of superficial and deep receptors in the contralateral forelimb and in the hindlimbs did not influence the RSCT units It is concluded that the receptive fields of the RSCT neurones are ipsilateral like those of other uncrossed tracts (Oscarsson 1964a) and limited to the forelimb

d Supraspinal control In the decerebrate preparation transmission in the FRA paths to motoneurones (Eccles and Lundberg 1959, Holmqvist and Lundberg 1959, 1961), primary afferents (Carpenter, Engberg, Funkenstein and Lundberg 1963), and several ascending tracts (Holmqvist, Lundberg and Oscarsson 1960) is tonically depressed This depression is due to a bulbospinal system which descends in the dorsal part of the lateral funiculus In experiments on decerebrate preparations (Oscarsson and Uddenberg 1964) the effects from cutaneous receptors were almost completely lacking in the RSCT units, suggesting that the FRA paths to this tract are likewise under inhibitory control The effects evoked from the FRA and described in this paper were tested on unanaesthetized preparations which were decapitated, or (2 expts) decerebrated and with the dorsal half of the cord transected in the first cervical segment The latter procedure would interrupt the descending pathways responsible for the bulbar inhibition of FRA paths (Holmqvist *et al* 1960) In the two experiments with intact ventral pathways (*e g* Exp III in Table I) FRA volleys evoked a discharge in the RSCT units more frequently than in the experiments made on spinal preparations (*e g* Exp I and II) The possibility of descending ventral pathways with a facilitatory action on the FRA paths should be considered

3 *Receptive fields of VSCT units*

Previous investigations had demonstrated that VSCT neurones receive FRA effects from bilateral receptive fields including the hindlimbs and hinder part of the trunk

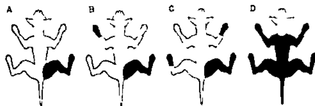
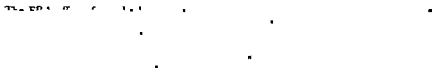


Fig 7 Areas supplying FRA excitation (hatched) and inhibition (black) to four VSCT units activated monosynaptically by Group I afferents in the contralateral (right) hamstring nerve

(Oscarsson 1957, Lundberg and Oscarsson 1962) It has now been found that these neurones receive FRA effects also from the forelimbs and front part of the trunk More than 50 VSCT units in 5 expts were identified by their monosynaptic excitation from Group I afferents in the contralateral hamstring nerve One experiment will be described in detail It was made on a decerebrate cat with the dorsal pathways interrupted in the first cervical segment Twelve VSCT units were encountered Ten of these responded with a train of action potentials on stimulation of all, or most of the forelimb nerves tested A typical example is shown in Fig 6 The number of spikes varied from one to more than 10 and the latency (measured from the stimulus artefact to the first spike) was usually between 10 and 20 msec (range 8—50 msec) On repetitive stimulation the VSCT neurones were usually excited but sometimes inhibited as shown by an increase or decrease in the frequency of the resting activity The two units which did not respond to single volleys were nevertheless excited or inhibited on repetitive stimulation

The FRA effects from the forelimbs were less readily displayed on natural stimulation of receptors Among the 12 units described above only five were appreciably influenced on pressure or pinching of the skin of the forelimbs The effects from the forelimbs were in general weak, they were always weaker than those evoked from the contralateral hindlimb but sometimes as strong as those from the ipsilateral hindlimb (*cf* Oscarsson 1957) The effects from the forelimbs were excitatory and/or inhibitory in various combinations Some examples of the receptive fields are shown in Fig 7



of effects has not been described previously Of the remaining 2 units one was excited from both hindlimbs and the other not influenced from either hindlimb

The effects from the forelimbs did not depend on pathways through the brain stem Similar effects were observed in the spinal preparations However, it is possible that descending ventral pathways from the brain stem facilitate the FRA effects (*cf* above) Natural stimulation of receptors in the forelimbs was observed to influence the VSCT units more often in the 2 expts with these pathways intact than in the three experiments made on high spinal animals

The effects evoked in VSCT neurones by stimulation of cutaneous receptors in the contralateral hindlimb were stronger than the effects evoked in RSCT neurones by

stimulation in the ipsilateral forelimb The significance of this finding is obscure The FRA paths to the RSCT neurones might depend more on facilitation from supraspinal centres than the corresponding paths to the VSCT neurones

Discussion

The conclusion that the RSCT is a functional equivalent of the VSCT (Oscarsson 1964b Oscarsson and Uddenberg 1964 1965) is supported by the present findings Both tracts are monosynaptically activated by high threshold Group I afferents which have been identified as tendon organ afferents The convergence of Group I excitation to individual neurones is as extensive in the RSCT as in the VSCT The patterns of convergence have been studied less in the RSCT than in the VSCT but the available evidence indicates that these patterns are similar in the two tracts There is often excitation from one synergic muscle group at each of several joints and sometimes from one group at each of the main limb joints Furthermore in both tracts co activation from antagonist muscle groups presumably does not occur though some observations on the RSCT might indicate exceptions to this rule The tract neurones are presumably activated from muscles that contract together in the execution of a certain movement or the maintenance of a certain posture (Oscarsson 1960, Eccles *et al* 1961) The RSCT and VSCT would carry information concerning stages of movement or position of the whole limb rather than information about change of tension in individual muscles

Both tracts receive FRA actions from large receptive fields In the RSCT the effects were relatively weak and predominantly excitatory whereas they were strong and predominantly inhibitory in the VSCT The difference in intensity of the effects in the two tracts might be related to a differential need for facilitation from higher centres The fact that the FRA effects are predominantly excitatory in the RSCT and predominantly inhibitory in the VSCT might be of little general significance Some VSCT units receive predominantly excitation in the cat (Oscarsson 1957 Lundberg and Oscarsson 1962) and excitation is the dominant effect in the VSCT of the dog (Oscarsson *et al* 1964)

The same problems arise in assessing the significance of the information forwarded by the RSCT because of its FRA connections as arise in connection with other ascending pathways influenced by the FRA It has been suggested that these tracts carry information concerning flexor reflex patterns for example by reflecting the degree of motoneuronal activation from interneurones on the paths from the FRA This would require that the interneurones transmitting effects from the FRA to motoneurones and to ascending tracts are influenced in a similar way not only from the periphery but also from higher centres (Lundberg 1959 Holmqvist *et al* 1960 Lundberg Norsell and Voorhoeve 1963) Experiments on the supraspinal control exerted by the pyramidal tract (Magni and Oscarsson 1961 Lundberg *et al* 1963) and by a bulbospinal system (Holmqvist *et al* 1960) demonstrate that this is indeed the case in some instances

With the RSCT there are obvious difficulties with the above hypothesis because of the weak effects from the skin and relatively strong effects on moderate stretch of muscle However these features might be due to the experimental situation in the intact animal with normal supraspinal control the FRA effects may be different With the VSCT this hypothesis might explain some observations made during the present investigation The VSCT neurones were shown to be influenced from the FRA not only in the hindlimb nerves but also in the forelimb nerves though the effects from the latter

were weak and variable The interneurones on the paths from the FRA in hindlimb nerves to lumbo-sacral motoneurones are influenced by the pyramidal tract and it has been suggested that the pyramidal effects on the motoneurones are exerted through facilitation and inhibition of segmental reflex arcs (Lloyd 1941, Lundberg Norsell and Voorhoeve 1962, Lundberg and Voorhoeve 1962) It is not unlikely that these interneurones are influenced also from the FRA in the forelimbs and at least in part responsible for the effects that these afferents exert on lumbo-sacral motoneurones (*cf* Lloyd 1942, Lloyd and McIntyre 1948) If the VSCT forwards information concerning the transmittability in these interneurones the effects from the forelimbs would be readily explained

This paper concludes, for the time being, our series of articles on the RSCT It has been shown that this tract is a forelimb equivalent of the VSCT, though a separate anatomical entity The RSCT and VSCT might have developed independently from different origins by parallel evolution of a similar pattern of organization (Oscarsson and Uddenberg 1965) The information forwarded by the two tracts is related to two systems of primary afferents the tendon organ afferents and the FRA Presumably the integrated information from these two systems is of special value for the cerebellum (Oscarsson 1960) and cannot be replaced by information through separate information channels for tendon organ and flexor reflex afferents Such channels are available in the DSCT and presumably also in the cuneocerebellar tract (Lundberg and Oscarsson 1960, Holmqvist, Oscarsson and Rosén 1963a)

Many problems concerning the RSCT remain to be solved The tract has not been identified with anatomical methods though such work is in progress (G Grant personal communication) Localization of the cell bodies and intracellular recording from them is desirable and would permit a detailed study of the synaptic properties and the patterns of convergence The organization of the VSCT has been investigated in several mammalian species (Magni and Oscarsson 1962, Oscarsson *et al* 1964) but the RSCT is known only from experiments on the cat Comparative studies might provide clues to the functional significance of the tract It is of interest that recent observations on the duck suggest that the RSCT and VSCT might exist in the bird and have a similar organization as in mammals (Oscarsson, Rosén and Uddenberg 1963)

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The in Vivo Effect of Angiotensin and Noradrenaline on the Proximal Tubular Reabsorption of Salt in Mammalian Kidneys

By

PAUL P LEYSSAC

Received 6 Oktober 1964

Abstract

Leyssac, P P *The in vivo effect of angiotensin and noradrenaline on the proximal tubular reabsorption of salt in mammalian kidneys* Acta physiol scand 1965 *64* 167—175 — The purpose of the investigation presented here was to study the immediate effect of angiotensin and of noradrenaline on the rate of proximal tubular reabsorption of filtrate in mammalian kidneys by a direct method By registration of the occlusion time, which is the time interval between interruption [illegible]

Clearance studies on the effects of noradrenaline (*e g* Jacobsen, Hammersten and Heller 1951, Smythe, Nickel and Bradley 1952, Berne *et al* 1952, Pullman and McClure 1954) and of angiotension (*e g* Book and Krecke 1958, Gross and Turrian 1959, Finnerty 1962) on salt excretion have shown that the renal response to these pressor substances is qualitatively identical, angiotensin being about 10 times more potent The typical acute effect in dogs and human subjects, a decrease in urine flow, sodium excretion, and renal blood flow with a more moderate reduction in the glomerular filtration rate, has generally been attributed solely to the vascular action of the pressor substances Since no correction for dead space error have been carried out in these clearance studies, in which a considerable reduction in urine flow occurred as a response to the administration of the pressor substances, a gross overestimation of the early reductions in glomerular filtration rate is introduced unless the initial one or two 10-min clearance periods are critically evaluated However, even with due respect to such likely gross errors it appears that at least during the initial 20—30 min of the experiments, before late secondary compensatory mechanism (*e g* aldosterone) may be

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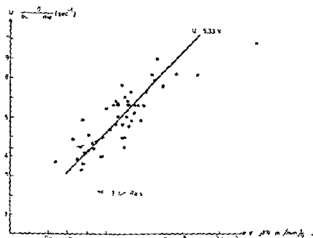


Fig 1 Reciprocal occlusion times (u) at spontaneously different rates of glomerular filtration (v) in control rats

in the kidneys and in the 10 samples of the injected dose were counted in a well scintillation counter connected to an impulse height analyser

Analyses of inulin were performed by the method of Bojesen (1952) modified for microanalysis

Results and calculations

Fig 1 shows the pooled control data of the reciprocal occlusion time (u) plotted against the means of 2—4 determinations of inulin clearance (v) measured immediately before the registration of occlusion time Included in the figure are also data published previously (Leyssac 1963) By the method of linear regression analysis, disregarding points with (v) > 1 6 ml/min/g KW because they are few and scattered the relationship between reciprocal occlusion time and the clearance of inulin was found to be represented by a straight line which did not differ significantly from a line described by (u) = 5 33 (v)

It appears that the spontaneous variation in clearance of inulin ranges between about 0 7 and 1 7 ml min/g KW with an accumulation of observations around 1 2 ml/min g KW Below this range of spontaneous variation a horizontal broken line has been drawn corresponding to the maximum occlusion time observed following the administration of 25 ng of synthetic angiotensin (Fig 2) Although not included in the statistical analysis a few observations at very high inulin clearances suggest a deviation from a direct proportionality between (u) and (v) or a systematic error in evaluation of one or both of these parameters at these high rates of reabsorption

The immediate effect of three doses of synthetic angiotensin on the reciprocal occlusion time at spontaneously different clearances of inulin is demonstrated in the Fig 2 to 4 It appears that an effect is clearly demonstrated at all three dose levels increasing with the dose administered until a maximum of occlusion time (about 23 sec) is reached by the injection of 25 ng of angiotensin This maximum equals the average maximal

operative, the absolute decrease in the rate of glomerular filtration of sodium by far exceeded the fall in sodium excretion, indicating a decreased rate of overall reabsorption. Since it has been demonstrated (Leyssac 1963) that the rate of proximal tubular reabsorption of salt (which normally accounts for about 75 per cent of the total reabsorption) is independent of the filtered load' eo ipso (i. e. that the proximal reabsorption is a transport process always operating at its T max, the level of which is physiologically variable), the change in the proximal tubular sodium T-max must have been due either to a direct action of the pressor substances or due to an indirect action mediated by a rapid intrarenal regulatory mechanism.

It was previously observed (Leyssac 1964) that angiotensin has an immediate and direct depressing effect on the proximal reabsorption rate (T-max), by which this rate may be reduced to a certain minimum level equal to the lowest value in the range of physiological variation. The purpose of the present investigation was to determine the dose of angiotensin necessary to depress the sodium T max to its minimum, and to investigate whether a similar direct tubular effect of noradrenaline could be demonstrated. The results have indicated that about 0.7×10^{-9} g (0.7 ng) of angiotensin acting per g of kidney is capable of eliciting an almost maximum depression of the proximal tubular reabsorption rate in rat kidneys of about 0.8 g. No immediate tubular effect of noradrenaline could be demonstrated, suggesting that its effect on the renal tubular handling of sodium is indirect and mediated by an intrarenal regulatory mechanism most likely involving the renin angiotensin system.

Methods

Male albino rats weighing about 250 g were anesthetized and prepared for determination of occlusion time and measurement of inulin clearances from the exposed left kidney as described in details elsewhere (Leyssac 1963, 1964). For testing the tubular effect of the two pressor substances either synthetic angiotensin (Val 5-angiotensin amide CIBA) or noradrenaline was given as a single intravenous injection immediately after the last collecting period for clearance determinations. At the very moment when the pressor substance reached the kidney, that is as the kidney began to pale, the aorta was totally clamped and the occlusion time was noted. Angiotensin was administered in doses either of 25 ng, 7 15 ng or 5 ng in 0.050 ml of saline. Noradrenaline was given in doses of 400 to 600 ng in 0.050 ml of saline.

In some experiments the occlusion time was registered immediately after the last urine collecting period without administration of pressor substance (controls). The tubules were then allowed to refill by opening the clamp, angiotensin or noradrenaline was given after an interval of about 2 min and the occlusion time was registered again. In other experiments after the administration of noradrenaline or angiotensin and the determination of occlusion time, the tubules were allowed to refill and 2 to 3 min after the first measurement of the occlusion time the aorta was clamped again and the occlusion time noted without giving any more pressor substance. These latter determinations of occlusion time are also included in the control determinations. When more than one injection of pressor substance was given to the same animal, subsequent injections were given after an interval of at least 15 min, in which time urine was collected for repeated clearance determinations.

In another series of experiments a single [illegible]

(0.15 mg 0.035 μC) in 0.0 [illegible]

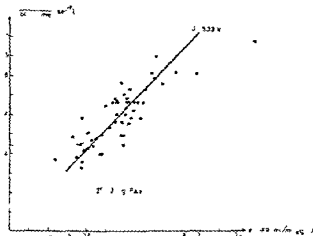


Fig 1 Reciprocal occlusion times (u) at spontaneously different rates of glomerular filtration (v) in control rats

in the kidneys and in the 10 samples of the injected dose were counted in a well scintillation counter connected to an impulse height analyser

Analyses of inulin were performed by the method of Bojesen (1952) modified for microanalysis

Results and calculations

Fig 1 shows the pooled control data of the reciprocal occlusion time (u) plotted against the means of 2—4 determinations of inulin clearance (v) measured immediately before the registration of occlusion time Included in the figure are also data published previously (Leyssac 1963) By the method of linear regression analysis disregarding points with (v) > 1 6 ml/min/g KW because they are few and scattered the relationship between reciprocal occlusion time and the clearance of inulin was found to be represented by a straight line which did not differ significantly from a line described by (u) = 5 33 (v)

It appears that the spontaneous variation in clearance of inulin ranges between about 0 7 and 1 7 ml min g KW with an accumulation of observations around 1.2 ml min/g KW Below this range of spontaneous variation a horizontal broken line has been drawn corresponding to the maximum occlusion time observed following the administration of 25 ng of synthetic angiotensin Fig 2 Although not included in the statistical analysis a few observations at very high inulin clearances suggest a deviation from a direct proportionality between u and v or a systematic error in evaluation of one or both of these parameters at these high rates of reabsorption

The immediate effect of three doses of synthetic angiotensin on the reciprocal occlusion time at spontaneously different clearances of inulin is demonstrated in the Fig 2 to 4 It appears that an effect is clearly demonstrated at all three dose levels increasing with the dose administered until a maximum of occlusion time about 23 sec is reached by the injection of 25 ng of angiotensin This maximum equals the average maximal

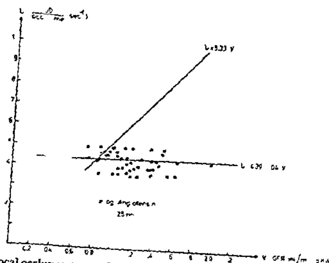


Fig 2 Reciprocal occlusion times after injections of 25 × 10 ⁸ g of angiotensin at spontaneously different rates of glomerular filtration measured immediately before the administration of angiotensin

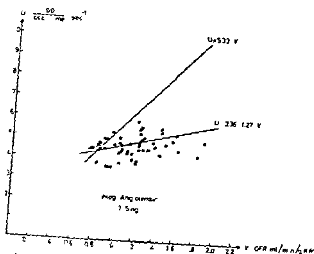


Fig 3 Reciprocal occlusion times after injections of 7 15 × 10 ⁸ g of angiotensin at spontaneously different rates of glomerular filtration, measured immediately before the administration of angiotensin

occlusion time observed spontaneously (Fig 1) which corresponds to an inulin clearance of about 0 85 ml/min/g KW

The indication of a maximal tubular effect caused by angiotensin suggests a dose-response relationship with horizontal asymptote A quantitative description of the observed effect derived from a relationship of this form, according to which the regression lines given in Fig 2—4 are calculated, will be published elsewhere It does, however, appear directly from the figures that the amount of exogenous angiotensin

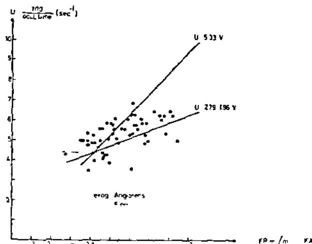


Fig 4 Reciprocal occlusion times after injections of 5×10^{-9} g of angiotensin at spontaneously different rates of glomerular filtration measured immediately before the administration of angiotensin.

necessary to reduce the inulin clearance of a rat kidney from 1 60 to 0 85 ml/min/g KW is about 20×10^{-9} g

By injection of ^{131}I labelled albumin with 10 ng of synthetic angiotensin in 0 050 ml of saline, removal and snap-freezing of the left kidney 9 sec after start of the injection and subsequent registration of the radioactivity in the kidneys and in 10 samples of 0 050 ml of the injected solution, the activity in the kidneys in per cent of the injected amount of activity was found to be

$$\frac{(419 \pm 30\ c/m)}{(13\ 450 \pm 250\ c/m)} \times 100 = 3\ \%$$

This fraction of the injected dose was independent of the concentration of pressor substances since identical values (2—4 per cent) was found no matter whether albumin was given alone or with angiotensin in doses from 10 to 25 ng or with 500 ng of nor adrenaline Assuming that also 3 per cent only of the injected amount of angiotensin was present in the kidney at the time of registration of its effect, and that the spontaneous variation in the rate of proximal reabsorption is determined by the concentration of endogenous angiotensin, the data indicate that the difference in the amount of endogenous angiotensin in a rat kidney, which may be responsible for the physiological interval of variation equals about 0 6 ng (0 3—0 9) or about 0 7 ng (0 35—1 0 ng)per g kidney weight, since the kidney weight of 250—300 g rats was found to be 0 87 ± 0 08 g Since the cortex constitutes the major part of the rat kidney (about 80 %) and has a blood flow per g of tissue at least not less than the medulla and since the tubular cells make up some 60 per cent of the cortical volume, it may also be assumed that some 1 2 ng of angiotensin (= 1.2×10^{-12} moles or about 7×10^{11} molecules) would be required per g of tubular cells to cause a maximal depression (by angiotensin of the glomerular filtration rate Assuming the cells to be cubic with a thickness of 6—7 microns about 200 molecules per cell would account for a depression of the filtration rate from the upper to the lower limit of the spontaneous range of variation

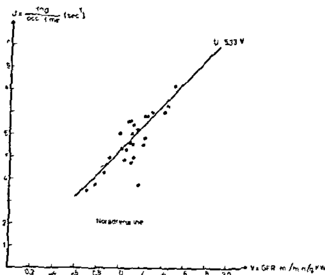


Fig. 5. Reciprocal occlusion times after injections of 4—6 × 10⁻⁷ g of noradrenaline at spontaneously different rates of glomerular filtration measured immediately before the administration of noradrenaline

Since pilot experiments with noradrenaline in doses of 100 ng failed to demonstrate any effect on the occlusion time, the present experiments were carried out with pressor doses of noradrenaline (400—600 ng) exceeding those of 25 ng of angiotensin, which did provoke an almost maximal effect. With both pressor substances the same fraction of the injected dose (2—4 per cent) may be assumed to have been present in the kidney at the instant of registration of the effect. It appears from Fig. 5 that even such heavy doses are without any effect at all on the occlusion time. The observed relationship between reciprocal occlusion time following noradrenaline and the (preceding) clearance of inulin does not differ from the regression line, (u) = 5.33 (v), observed when no drugs were administered.

Discussion

A. The relationship between the rate of proximal reabsorption and the glomerular filtration rate

In continuation of previous results (Leyssac 1963) the pooled control data have confirmed a linear relationship between the reciprocal occlusion time, representing the rate of proximal reabsorption and the inulin clearance within the range of physiological variation. However, at rates of filtration well below this range of spontaneous variation a deviation from the linear relationship was observed in partially clamped kidneys

similar maximum inhibition on the proximal salt T-max was caused by endogenous angiotensin as that elicited by exogenous angiotensin. In Fig. 1 this maximum inhibition is indicated by the interrupted line at filtration rates below 0.8 ml/min/g KW

B *The tubular response to angiotensin*

Evidence was presented previously (Leyssac 1964) that the injection of a high dose of angiotensin had not to any significant degree affected the luminal diameter of the proximal tubules at the moment of clamping the aorta (i. e. 9 sec from start of the injection). It was further stated that the instantaneous renal vasoconstriction induced by the substance, on principle being equal to the interruption of the renal circulation by clamping the aorta or the renal artery, did not itself affect the proximal rate of reabsorption as evaluated from the occlusion time. This statement is quite established by the present demonstration that doses of noradrenaline with a reno-vasoconstrictive effect exceeding that of doses of angiotensin causing maximal inhibition of the reabsorption rate does not influence the rate of reabsorption. The dose-dependent depression of this rate, as demonstrated following injections of angiotensin must therefore be due to a direct tubular effect in accordance with previous conclusions.

By simultaneous injection of labelled albumin evidence is presented that only a minor fraction (about 3 per cent) of an injected dose has actually reached the kidney at the moment of clamping the aorta. Since the vasoconstriction (and hence the paling) is almost instantaneous and since the aorta is clamped at the very moment when the kidney starts to pale, it is unlikely that either labelled albumin or exogenous angiotensin has left the kidney by the venous route at the instant of freezing. Disregarding a possible overestimation in assuming that all of the molecules present in the kidney has actually reached the effector sites at the moment of registration of their effect, the results indicate that a maximum of change in the proximal salt T max may be elicited by a change in the amount of angiotensin of about 0.6 ng per rat kidney. Since the kidney is highly sensitive to a change of this order of magnitude, one would expect that the endogenous amount of free angiotensin in the kidney is of a similar order of magnitude. The present data therefore suggest that an order of magnitude of some hundred molecules of angiotensin per cell is sufficient to elicit a maximum physiological depression of the rate of proximal tubular reabsorption. This reasonably low value would seem to support the previous conclusion (Leyssac 1964) that angiotensin is a physiological regulator of the proximal salt T max.

C *Interpretation of results obtained with angiotensin in clearance studies*

Different or even directly opposing tubular effects of angiotensin has been concluded from indirect evidence obtained in clearance studies. In accordance with the ideas of Smith (1951) the "typical" response, i. e. reduced excretion rate of sodium, to renin, angiotensin or noradrenaline in dogs and normal human subjects was generally ascribed to the vasoconstrictive action of the pressor substances or, based upon it seems most insignificant evidence, to an increased sodium reabsorption (Biron *et al.* 1962, Laragh *et al.* 1963). In contrast to the "typical" response to angiotensin or noradrenaline a marked increase in sodium excretion rate in response to these substances was demonstrated in rabbits and rats (Pickering and Prinzmetal 1940, Huges-Jones *et al.* 1949, Eversole, Giere and Rock 1952, Peters 1963), in hypertensive humans (Peart and Brown 1961, Biron *et al.* 1962), and in patients with cirrhosis and ascites (Laragh *et al.* 1963). Renal plasma flow was clearly decreased, whereas changes in glomerular filtration rate were less significant. The response in these species of animals and in patients with hypertension or secondary aldosteronism was ascribed to an inhibition of tubular sodium reabsorption. Taking into account the T max character of the reabsorp-

tion and the very small amount of sodium excreted per min relative to the rate of glomerular filtration of sodium these conclusions would seem unjustified irrespective of inevitable errors in the clearance measurement Even disregarding such errors, the clearance data presented (*e g* Biron *et al* 1962) show that the absolute amount of sodium reabsorbed per unit time was reduced following the administration of angiotensin not only in hypertensive patients but also in normal subjects Thus it is more likely that angiotensin has exerted a depressing effect on the rate of reabsorption (proximal T-max of salt) in both groups According to this interpretation the different effects on the rate of sodium excretion observed in the two groups of patients and between different species of animals, differences of no more than a few hundred μeq Na per min, corresponding to a few ml of glomerular filtrate, would be explained by the combination of slightly different arteriolar responses to angiotensin in the two groups with a similar (maximal) depressing effect on the proximal reabsorption rate *i e* a condition of disturbed glomerulo-tubular balance" conditioned by the combined vascular and tubular effect of angiotensin The difference in vascular response should only account for a difference in filtration pressure sufficient to change filtration rate a few ml per min all other factors being equal A decreased pressor response to angiotensin was demonstrated in experimental secondary aldosteronism (Davis, Carpenter and Ayers 1962) and a change in the excretion rate of sodium in response to angiotensin was also demonstrated in patients with secondary aldosteronism and in hypertensives, in whom the excretion of aldosterone may often be somewhat increased (Laragh, Cannon and Ames 1963, Genest 1963) In hypertensive patients other factors than aldosterone may possibly further change the vascular response to angiotensin

In this context it should be emphasized that the filtration rate was reduced in normal subjects during infusions of angiotensin from control values of about 120 ml/min to about 80 ml/min In hypertensive patients control values of about 80 ml/min (still within the normal range) only changed to about 75 ml/min during the infusion (Biron *et al* 1962) A similar reduction from 120 to 80 ml/min in normal subjects was reported by Finnerty (1962) These observations agree with the present direct evidence of a maximum effect of angiotensin on the proximal salt T max, which reduces the T max to the minimum value observed spontaneously

This work was supported by grants from Statens almindelige Videnskabsfond the Novofond and Kong Christian den Tiendes Fond for which the author wishes to express his gratitude

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From the Institute for experimental Medicine, University, Copenhagen, Denmark

The Luminal Occlusion Time of Proximal Tubules in Kidneys of Young Rats

By

PAUL P LEYSSAC

Received 6 October 1964

Abstract

Leyssac, P P *The luminal occlusion time of proximal tubules in kidneys of young rats* Acta physiol scand 1965 64 176—181 — The purpose of this investigation was to test the validity of the luminal occlusion time as a method for estimation of the rate of proximal reabsorption The [illegible]

far less than in kidneys of adult rats

Measurements of the luminal occlusion time, defined as the time required from interruption of the renal circulation and filtration until the proximal tubules have completed reabsorption of the luminal filtrate, has been used as a relative estimate of the volume of proximal fluid reabsorbed per unit time and unit tubular length, i e of the rate of proximal reabsorption (Leyssac 1963) Provided that the volume of fluid per unit tubular length of the proximal tubules (i e the internal diameter) is kept reasonably constant from one experiment to the other by using animals of the same body weight and kidney weight, in which the number and dimensions of the proximal convoluted segments should be equal, a difference in the occlusion time should directly indicate a difference in the rate of transcellular transport of salt or salt T-max

The validity of the occlusion time method as a measure of the proximal reabsorption rate was based mainly on the demonstration by Hanssen (1960) that during the luminal occlusion filtered ferrocyanide was neither displaced to any significant degree towards the distal part of the nephron nor in direction of the glomerulus Supporting evidence was given by the close accordance between the measured occlusion time and the expected time necessary to empty the proximal lumina, as calculated from the rate of glomerular filtration and the dimensions of the proximal tubules (Leyssac 1963)

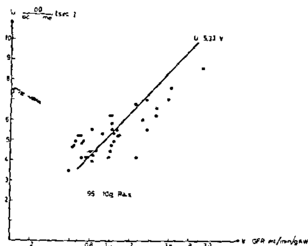


Fig 1 Reciprocal occlusion time at spontaneously different rates of glomerular filtration in 100 g rats The regression line u = 5 33 v was calculated from similar observations in 250—300 g rats

As an attempt to verify the validity of the method, the purpose of the present investigation was to see whether the close correspondance between the rate of reabsorption as evaluated from the measured occlusion time and the immediately preceding clearance of inulin holds also in cases in which the volume of fluid per unit tubular length was different from that of previous experiments

After the earliest post embryonic stages the growth of the kidney depends solely on the growth of the tubules which predominantly is due to an increase in the size of the proximal segment as shown by Sperber (1944) in maceration specimens Measurements in vivo by Steinhausen *et al* (1963) of the internal diameters of proximal tubules have further shown that the diameters of adult rats are larger than those of young rats Therefore in the present investigation the relationship between reciprocal occlusion time and rates of glomerular filtration was studied in the range of spontaneous variations in young rats and the internal diameters and cellular areas of the proximal tubules were measured It is shown that the relationship between occlusion time and rate of filtration per g kidney weight (KW) does not differ significantly from that observed previously in adult animals (Leyssac 1963 1965) in agreement with expectancy from the measured diameters and cellular areas

Methods

Male albino rats weighing 90—100 g were anesthetized and prepared for determination of occlusion time and measurement of inulin clearances from the exposed left kidney as described previously (Leyssac 1963 1964

In another series of experiments the left kidney was quickly removed instead of clamping the aorta and frozen within one sec from removal at — 160 C in isopentane cooled with liquid nitrogen The frozen kidneys were transferred to absolute ethanol at — 20 C for freeze substitution and thereafter embedded in paraffin sectioned (3 μ thickness and stained with periodic acid Schiff (MacManus Histological sections for measurement of internal diameters

TABLE I. Shows the ± stand. deviations of the mean of kidney weights and internal diameters measured in the two groups of rats. Numbers in brackets give the range of values. The number above the mean value gives the number of kidneys examined.

Body weight	Kidney weight	Luminal Diameter	Calculated Luminal area
	26	6	
95—110 g	0.429±0.096 g	19.0± 3.2 μ	284 μ^2
		(17.3—22.2)	
	100	6	
250—200 g	0.874±0.079 g	27.1± 1.7 μ	573 μ^2
		(25.4—28.8)	

were prepared from two groups of rats with body weights of 90—110 g and 250—300 g respectively. The internal diameters and cellular areas of the proximal tubules were measured by means of a micrometer ocular and planimetry respectively in 50 circular (*i. e.* transversely cut) proximal tubules from the superficial cortex of each kidney.

Results

In the figure the reciprocal occlusion time (u) is plotted against the means of 2—4 determinations of inulin clearance (v) measured immediately before the registration of occlusion time. In the figure is also given the regression line (u) = 5.33 (v), calculated from data obtained in experiments on adult rats (Leyssac 1965). It is seen that the observations registered in the small rats are distributed along this regression line, which passes through (0.0).

The line through (0.0) best fitting the present observations, disregarding observations with (v) > 1.60 ml/min/g KW, has a slope of $\bar{u}/\bar{v}$ = 5.32, *i. e.* its equation (u = b(v)) is u = 5.32 (v) with a variance of y (s^2) = 0.965 and a variance of b (s_b^2) = 0.016 and 39 degrees of freedom. This line does not differ significantly from the line u = 5.33 (v) found for adult animals in the main interval of physiological variation.[1]

Since the number of nephrons does not increase with growth after the early post natal period (Sperber 1944), a certain glomerular filtration rate (GFR) per g of kidney weight (KW) represents in small rats (with an average kidney weight of 0.43 g) a filtration rate per nephron about half of that of adult kidneys (of a kidney weight of 0.87 g).

From the data given in the table it appears that the transsectioned proximal luminal area in kidneys of young rats was about half of that in adult rat kidneys. Consequently the volume of tubular fluid per unit tubular length in the small rats was also half of the luminal volume in adult rats. Since furthermore the kidney weight in the small animals was also half of that in adult rats, it follows that the volume of fluid per unit tubular length is approximately proportional to the kidney weight.

The ratio cellular area to luminal area, which was found to be on an average 1.18 in small rats and 1.05 in adult rats, did not differ significantly (p > 0.05).

[1] The statistical analysis was carried out by Inger Harder Hansen, to whom the author is deeply indepted.

Discussion

By the present technique of snap-freezing and histological procedure, which was adopted from the investigations of Hanssen (1960), it seems possible to obtain a histological picture of the renal cortex, which corresponds, with some reservations, rather closely to the conditions in vivo From the internal diameters of transsectioned proximal tubules it is calculated that the luminal area in 100 g rats is about half of the luminal area of proximal tubules in rats weighing 250—300 g, in close agreement with the *observations in vivo reported by Steinhausen et al (1963) Also the present absolute* values of proximal internal diameters in the two groups of rats (19 μ and 27 μ respectively) agree reasonably well with those measured by Steinhausen *et al*, as they found that the proximal internal diameters in vivo were 16—17 μ in 80 g rats and 23 μ in 300 rats Thus the luminal diameters measured in the histological sections of both groups of rats were slightly larger than those observed in vivo The small discrepancy may in part be due to differences in the experimental conditions (degree of hydration, etc), but it can, in the main, be accounted for by the fact that the brush border was included in the present measurements, because it is less well-defined in the histological sections whereas this was not the case in the in vivo measurements

The real luminal volume should probably be calculated from a value between these two extremes

If the total luminal volume of the proximal convoluted segment is denoted by V the volume of fluid reabsorbed per sec in this part of the proximal tubule during the luminal occlusion by V, and the occlusion time by occl t we have that

$$V = V \times \text{occl t.} \tag{1 a}$$

or

$$\frac{1}{\text{occl t}} = \frac{V}{V} \tag{1 b}$$

If we denote the fraction of filtrate normally reabsorbed by the convoluted proximal segment by F, and make the assumption that the reabsorption continues in this segment at an unaltered rate after interruption of the renal circulation and filtration we also have that

$$\frac{1}{\text{occl t}} = \frac{F}{V} \times \text{GFR} \tag{2 a}$$

or

$$\frac{1}{\text{occl t}} = \frac{F}{\left(\frac{V}{KW}\right)} \cdot \frac{\text{GFR}}{KW} \tag{2 b}$$

Thus the relationship between reciprocal occlusion time and GFR per g KW should remain unchanged with equal values of F and $\frac{V}{KW}$ The present data indicate that this relationship does not differ significantly in young and adult rats (equal slope — equal $\frac{F}{\left(\frac{V}{KW}\right)}$) If it may be assumed that the fractional reabsorption of filtrate in the proximal convoluted segment F) is approximately the same in young and adult rats which seems reasonable $\frac{V}{KW}$ should be equal in the two groups of rats investigated if the

general assumption of unaltered rate of reabsorption during the luminal occlusion was valid. Taking into account that the proximal tubules constitute the major fraction of the kidney weight, $\frac{V}{KW}$ actually seems to be equal in young and adult rats, since the ratio luminal area to cellular area was found to be approximately equal in the two groups. The present data, therefore, support the previous conclusion that the proximal reabsorption continues at an unaltered rate after cessation of filtration (Leyssac 1963).

The rather closely coinciding completion of the luminal occlusion of all the tubules in a field of vision indicates that all visible parts of the proximal convolutions, equal to the first two thirds of this segment, complete reabsorption almost simultaneously (within 4—5 sec). Since it was demonstrated by Hanssen (1960) that the proximal fluid is not to any significant degree displaced either distally or in direction of the glomerulus following interruption of the circulation, a well-defined end point of occlusion of the whole visible fractions of these segments indicates a very uniform reabsorption rate throughout this part of the tubules. This conclusion is in disagreement with some interpretations of recent micropuncture data. Lassiter, Gottschalk and Mylle (1961) suggested a linear relationship between proximal tubular fluid to plasma ratios of inulin and the distance of collection from the glomerulus from results obtained in non diuretic rats, which would indicate a rapidly decreasing rate of reabsorption along the convoluted segment. Because of a wide scatter of observations a linearity was, however, not established. In later reports by Lassiter, Mylle and Gottschalk (1964) and by Giebisch, Klose and Windhager (1964) on saline diuretic rats the observed proximal inulin ratios were said to be consistent with a model presented by Kelman (1962). In this model the proximal tubule is considered to be analogous to a catalytic flow reactor, in which reaction velocity or rate of fluid reabsorption per unit length of the proximal tubule is proportional to the rate of fluid flow through the tubule, the flow of which therefore decreases exponentially with the distance from the glomerulus. This model, thus, is in general agreement with the ideas of Smith (1951). This model requires that a semilogarithmic plot of the inulin ratios against distance from the glomerulus should yield a straight line passing through (1.0). This was however apparently not the case. In both reports the data may be fitted to a straight line, but this line clearly intercepts with the ordinate (TF/P inulin) at a value significantly lower than unity. The reported data, therefore, are obviously incompatible with an exponentially decreasing reabsorption rate along the proximal convoluted segment but seem to fit very closely the theoretical curve calculated from the assumption of equal reabsorption rates along this segment of the tubules. Also the linear relationship between percentile proximal fluid reabsorption and the distance from the glomerulus calculated from the data of Walker *et al.* (1941), and from the observations by Windhager and Giebisch (1961 Fig. 3) and by Giebisch, Klose and Winhager (1964, Fig. 1) indicate a uniform reabsorption rate along the proximal convolutions. Thus, micropuncture data as well as the well-defined end point of the luminal occlusion further support the concept that the rate of proximal reabsorption is independent of the "filtered load", *i.e.* that it has the character of a T max process, as suggested by Bojsen (1954) and indicated by previous results from this laboratory (Leyssac 1963).

This work was supported by grants from Statens almindelige Videnskabsfond, Novofonden and Kong Christian den Tiendes Fond for which the author wishes to express his gratitude.

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Fig. 1 A. Experimental set up for administering a constant concentration of $^{14}CO_2$ to spontaneously breathing rats. A given amount of $Na_2{}^{14}CO_3$ (200 μc) was pipetted into a 50 ml glass flask (df) equipped with 2 side arms and a glass stopper. The flask was connected to the interior of a 12 litre Buchner flask (def) via a short glass tube fitted into a rubber stopper in the 12 litre flask. In order to obtain gaseous $^{14}CO_2$ in the delivery flask (def), acid was injected through a rubber stopper in a side tube in the diffusion flask (df) and the content of the latter flask was stirred magnetically. The attainment of a diffusion equilibrium (controlled with continuous measurement of the $^{14}CO_2$ activity in the flask by means of a thin window GM tube) was aided by means of a fan inside the delivery flask (fm). After completed diffusion a clamp was opened on a rubber tubing connecting the delivery flask (def) with a reservoir flask (rf) containing glycerol. The radioactive gas mixture which was displaced by the glycerol flowed through a rubber tubing from the delivery flask (def) to a fan. The rats were connected to the gas mixture by joining directly the tracheal cannula to a T tube in the outlet rubber tubing. B. Diffusion unit for absorption of the $^{14}CO_2$ in the tissue into Hyamine 10X. The frozen and crushed tissue was added to the main compartment of the unit (mf) made from a 50 ml glass stoppered flask. The flask contained 3 ml of a 10 per cent trichloracetic acid. The content of the flask was stirred magnetically for 2 hours and the carbon dioxide evolved was captured into 0.3 ml of Hyamine 10X, contained in a small cup in a side tube (st).

In the other series of experiments rats weighing between 150 and 160 g were used. In this group one animal was killed every 15 min of an exposure whose final length was 120 min and the $^{14}CO_2$ content in the acid labile, the acid soluble, the protein, the lipid and the nucleic acid tissue fraction was determined.

All animals were anaesthetized with Nembutal (3—4 mg/100 g b. w.), tracheotomized and allowed to breathe spontaneously. The animals were left undisturbed for 15 min before they were exposed to the radioactive gas mixture. In experiments with long exposure times the animals sometimes began to recover from the anesthesia and reacted to painful stimuli. In such cases an additional dose of Nembutal was given (1 mg/100 g b. w.). All rats with respiratory depression or with irregular breathing were rejected. During the exposure period the head of the rat was secured with a head holder while the body rested on a metal box which could be heated with a lamp so as the minimize heat losses.

After each exposure period the rat was disconnected from the radioactive gas mixture and the head was quickly frozen by immersion into liquid nitrogen. When the head [illegible] s completely frozen it was sawed into slices about 5 mm in thickness [illegible] h exchange able blades. In order to prevent [illegible] on the saw [illegible] The brain and the [illegible]

tissue was then separated from the skull by pressing with the point of a chilled surgical blade at the margin of the bony coverings This procedure usually left the dura intact but it could easily be removed with the help of a surgical blade Two of the separated brain tissue slices were usually used to prepare cortical samples, which were split off 1—2 mm from the surface of the frozen tissue

Each animal was exposed to a constant concentration of $^{14}CO_2$ in the inspired air This was

Muller tube connected to a rate meter and an ink writer It was found that a constant activity was obtained after a diffusion time of about 2 5 hrs and that the activity remained unchanged even after 7 hrs In each subsequent experiment a diffusion time of 4 hrs was allowed for before the rat was connected to the gas supply

After completed diffusion the clamp on the outlet tube from the delivery flask (def) was

rat was then connected to the radioactive source by joining the straight tracheal cannula with a T branch on the outlet tube

1 4-di [2 (5-phenyloxazole)] benzene (POPOP) per litre of toluene (p.a) The glass cups containing the Hyamine 10-X were transferred to the counting vials and 5 ml of the scintillation mixture was added to each vial The vials were then refrigerated overnight and the samples subsequently counted in a Panax liquid scintillation equipment Before counting the glass cups were carefully emptied and removed The samples were counted for 10^4 seconds with an E.H.T of 1400 V and a discriminating voltage of 10 V A blank was run for each experiment For this purpose 0.2 ml of a 0 01 M Na_2CO_3 solution was added to the trichloracetic acid in a diffusion unit and the sample was then treated identically with the tissue sample The blank

count was subtracted from the tissue count and the latter was then calculated as the number of counts per gram of tissue

Before further handling of the samples the acid-soluble and the nucleic acid fractions were extracted several times with ether to remove most of the trichloracetic acid These two fractions as

50

10-

remained In the whole tissue sample and in the protein sample a few tiny strands of tissue

mended

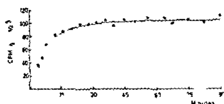
When the samples had been digested they were diluted with 2 4 dioxane up to a final volume of 10 ml Aliquots (2 ml) of the resulting solutions were then added to the counting vials together with 5 ml of scintillation mixture The latter was prepared with PPO POPOP and

of 1 > \ The samples were counted for a least 10^4 sec and the background was corrected for by passing a blank sample through the whole procedure An internal standardization was also used in that aliquots of a standardized hexadecane 1-C-14 solution (The Radiochemical Centre Amersham England) was added to each sample with subsequent recounting The

Results

A The equilibration of the administered $^{14}CO_2$ with the acid-labile carbon dioxide of brain tissue

In a previous publication results were presented to show that inspired $^{14}CO_2$ is rapidly incorporated into the acid labile CO_2 fraction of the brain equilibrium being reached in about 60 min (Siesjo 1963) In order to allow exposure periods up to 120 min rats weighing between 150 and 160 g were then used for the longer exposure times In the present material however, which comprises animals from the older material as well as new ones, all rats weighed around 200 g (190—210 g The effective volume of the delivery flask (see Fig 1) i e the volume of gas which could be displaced by the glycerol, was slightly more than 11 litres With the long exposure times (90 min or less) the rate of flow of gas in the outlet tube was thus about 125 ml per minute This volume exceeds by about 20 per cent the tidal volume of rats of the present size (Drorbaugh 1960)



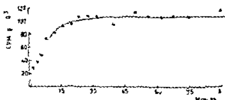


Fig 2 Fig 3

Fig 2 (left) The rate of equilibration between inspired $^{14}CO_2$ and the diffusible, acid labile carbon dioxide fraction of brain tissue The radioactivity is expressed as the number of counts per gram of tissue per minute Each point represents one experiment The curve drawn through the points follows the function $y = 1.05 \times 10^{5} (1-0.614\ e^{-0.28t}-0.518\ e^{-0.075t})$

Fig 3 (right) The rate of equilibration between inspired $^{14}CO_2$ and the diffusible acid labile CO_2 fraction of cortical tissue The curve drawn through the points follows the function $y = 1.08\ 10^{5}\ (1-0.581\ e^{-0.28t}-0.672\ e^{-0.105t})$ Note that the diffusible carbon dioxide fraction of cortical tissue comes into equilibrium with the $^{14}CO_2$ at a faster rate than does that of the whole brain

The specific activity of the radioactive $^{14}CO_2$ released into the system was so high that the resulting CO_2 tension in the gas phase never exceeded 1 mm Hg This tension could be assumed to be without detectable physiological effects

The rate of equilibration between the inspired $^{14}CO_2$ and the acid labile fraction of the tissue is illustrated in Fig 1 where the radioactivity of the brain tissue samples expressed as number of counts per gram of tissue per minute has been plotted against the time of exposure It is seen that with the type of rats used an apparent equilibrium was reached after about 45 min after which time no significant increase occurred in the activity

The corresponding curve for the cortical tissue is shown in Fig 2 If this curve is superimposed upon that of Fig 1 it can be seen that although the absolute figures at equilibrium are comparable to those for the whole brain the rate of uptake of $^{14}CO_2$ appears to be somewhat more rapid in the cortical tissue

An attempt was made to find equations which would approximately describe the rate of uptake of $^{14}CO_2$ as illustrated in Fig 1 and 2 It was found that no single exponential function fitted the experimental points The curve was therefore analysed according to the procedure described by Solomon (1949) In order to increase the accuracy however the logarithm of the experimental values in the last part of the curve (log y) was plotted against time (t) and the slope of the resulting curve was determined in a number of points with the help of a dernometer (Arner 1964) When the values for dy/dt were plotted against the corresponding values for y a straight line could be drawn through a number of points The slope of this line gave the coefficient k_2 in the equation below and by inserting this value into the equation $dy/dt = c_2 k_2 e^{-k_2 t}$ the corresponding value for c_2 was obtained The exponential term resulting was then subtracted from the original curve in the usual way (Solomon 1949) yielding a second exponential term No attempt was made to derive a third exponential function from the experimental curve

The rate of uptake of $^{14}CO_2$ into the acid labile CO_2 fraction of brain tissue could be approximately described by the equation $y = 1.05 \times 10^{5} (1-0.614\ e^{-0.28t} - 0.518\ e^{-0.075t})$ where y is expressed as counts min g of tissue It can be seen from the equation

TABLE I. Incorporation of $^{14}CO_2$ (dpm/g of tissue) into the acid labile (diffusible), the acid soluble, the protein, the lipid and the nucleic acid tissue fractions of brain tissue The last column denotes the recovery of the $^{14}CO_2$ in the different fractions as compared to the total $^{14}CO_2$ measured in the unfractionated tissue

Exposure time in min	Disintegrations/min/g of tissue						
	T Total	D Diffusible	AS Acid Soluble	P Protein	L Lipid	NA Nucl Acid	D+AS+P+L+NA/T %
15	110 400	99,300	7,300	1,800	500	500	99 1
30	153,600	128,700	16,800	2,000	500	600	96 7
45	172,000	141,600	27,400	2,300	600	1,200	97 2
60	196,800	157,200	30,600	2,900	600	1,100	97,8
75	195,000	151,800	34 100	3,200	800	1,800	98 3
90	222,000	164,100	53,400	5,200	2,100	1,600	102 0
105	231,600	162,600	54 300	4,200	1,400	1,400	96 7
120	255,500	164,400	75,000	6,600	2 300	2,600	98 2

that the sum of the constants c_1 and c_2 exceeds unity, implying that the curve starts with a certain time lag

A similar analysis was performed of the rate of incorporation of $^{14}CO_2$ into the cortical tissue This analysis could not be performed with the same accuracy due to the scatter of the experimental values An approximate fit was given by the equation $v = 1.08 \times 10^4 (1 - 0.584\, e^{0.28t} - 0.672\, e^{0.105})$

A comparison between the two equations indicated that the incorporation into the acid labile fraction of cortical tissue did occur at a faster rate than that into whole brain tissue and that the difference was due to the second, slow exponential term (see discussion)

B *The equilibration of the administered $^{14}CO_2$ with the acid-labile, the acid soluble, the protein, the lipid and the nucleic acid tissue fractions* In this series of experiments smaller rats were used (see Methods) and it became apparent that with these rats the time needed for an equilibrium to be reached between the administered $^{14}CO_2$ gas mixture and the acid labile CO_2 fraction of the brain was slightly longer (*cf* Siesjo 1963) The emphasis in this series was, however, laid on a comparison between the rate of incorporation of $^{14}CO_2$ into different tissue fractions. The relation between these rates are illustrated in Table I where the counting rates obtained with the different fractions have been compiled together with the per cent recovery

The total $^{14}CO_2$ activity in the tissue, including the acid labile and the different organic fractions, increased roughly exponentially during the first 60 min, after which time the increase was approximately linear The exponential increase during the first hour was given by the acid labile fraction (CO_2, H_2CO_3, HCO_3^-), which reached an *apparent* equilibrium during that time, while the sum of labelling of the different organic fractions (As + P + L + NA) was labelled at a linear rate during the whole exposure period

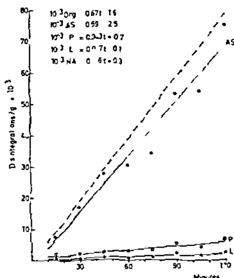


Fig 4 The rate of equilibration between inspired $^{14}CO_2$ and the organic fractions of the tissue The individual points for the total $^{14}CO_2$ incorporation into organic frac [illegible]

omitted since they correspond very closely to those for the lipid fraction (L)

The relation between the rate of incorporation of $^{14}CO_2$ into the different organic fractions is illustrated in Fig 4, where the equations for the straight lines have been calculated in order to facilitate comparisons The curve with the steepest slope represents the $^{14}CO_2$ activity in all the organic fractions If this is compared to the total $^{14}CO_2$ activity in the tissue (all the organic fractions – the diffusible, acid labile fraction) it is found that after 15 30, 60, and 120 min the organically fixed $^{14}CO_2$ in the tissue amounts to 8, 13, 21 and 32 per cent of the total activity, respectively The main part of the organic' $^{14}CO_2$ was recovered in the acid soluble fraction (about 85 per cent) while most of the remaining activity was found in the protein fraction (8—9 per cent) The lipid fraction and the nucleic acid fraction were equally labelled but the activity in these fractions was too low to allow any quantitative comparisons with the other fractions Thus, in none of these fractions did the $^{14}CO_2$ activity ever amount to one per cent of the total tissue activity It should be pointed out however, that the radioactivity in these fractions appeared to increase steadily with increasing time of exposure

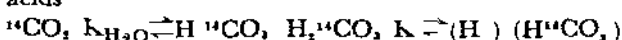
Discussion

As a first approximation it might be assumed that the laws governing the uptake of inert gases should also apply to CO_2 According to these laws which have been competently described (Morales and Smith 1948 Jones 1950 Kety 1951, *cf* also Severinghaus 1964) the rate of uptake of a gas in a tissue is determined by 3 main factors the lung ventilation the solubility of the gas in the blood and the blood flow to the tissue However even if it could be assumed that CO_2 fits qualitatively into these schemes, its peculiar characteristics make it adventurous to apply any quantitative relations formulated for other gases to CO_2 equilibria Thus, CO_2 is not only dissolved in the blood and in the tissue but also chemically bound, which implies that its absorption in the

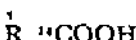
different phases does not follow Henry's law rather different carbon dioxide absorption curves. *Moreover, the gas is not physiologically inert but influences such parameters as* the lung ventilation and the blood flow to the brain. This is to say that the accumulation and the elimination of CO_2 leads to physiological changes, which in their turn influence the rate accumulation and the rate of elimination respectively (*cf.* Landau 1963). Finally, the time taken for the diffusion of the gas in the tissue cannot immediately be neglected since the *rate-determining diffusion coefficient incorporates a factor for the* binding of CO_2 in the tissue (Siesjö and Thews 1962).

It is clear that some of the difficulties encountered when studying the kinetics of CO_2 transients may be avoided by using the radioactive compound. Thus, the concentration of the administered gas can be kept so low that the physiological parameters do not change appreciably. The curve for the incorporation of $^{14}CO_2$ into the acid labile CO_2 of the tissue should then illustrate the basic rate of accumulation. Any moderate increase of the CO_2 concentration in the inspired gas mixture should by virtue of its effect upon the lung ventilation and upon the tissue blood flow tend to increase to rate of incorporation.

There is however a complication to this simple scheme which concerns the equilibrium between the inspired $^{14}CO_2$ and the total carbon dioxide content of the tissue. Thus the $^{14}CO_2$ coming to the tissue does not only exchange with the bicarbonate pool of the tissue but also with the carboxyl groups of free and protein bound organic acids.



$$^{14}CO_2 + H_2O \rightleftharpoons H_2{}^{14}CO_3 \quad H_2{}^{14}CO_3 \rightleftharpoons (H^+)(H^{14}CO_3^-)$$



$$\downarrow$$
$$R\text{-}^{14}COOH$$

The incorporation of $^{14}CO_2$ into organic compounds in the brain proceeds at a rate which is linear with time during the period studied presently. This means that the $^{14}CO_2$ concentration should be constantly below its equilibrium value in the present experiments and that the acid labile carbon dioxide fraction has not reached a true equilibrium during the 90 min studied in the first series of experiments. However since the amount of $^{14}CO_2$ which is incorporated per minute into organic compounds is less than 0.5 per cent of the total acid labile $^{14}CO_2$ at equilibrium and since the rate of diffusion of CO_2 is high (Siesjö and Thews 1962) the $^{14}CO_2$ activity in the present experiments should be rather close to the true equilibrium values.

The present work was undertaken to investigate the possible presence of an active transport of hydrogen ions between brain tissue and blood plasma. Such a transport may be approached from kinetic and from physicochemical angles. In the present context only the kinetic approach will be touched upon while the physico-chemical arguments will be left for further treatment (Siesjö 1964). The present work has shown *that if rats are exposed to a gas mixture with radioactive* CO_2 *the diffusibly* carbon dioxide fraction of the brain (CO_2, H_2CO_3 and HCO_3^-) comes into an apparent equilibrium with the inspired $^{14}CO_2$ within an hour or less. According to available theory (see above) this equilibrium would be reached much quicker if the CO_2 tension of the inspired gas mixture was increased. However, in the work of Nichols (1958) and in experiments from the laboratory (Siesjö 1964) the apparent equilibrium time was not shorter than the present one but 3 times as long (see introduction). The discrepancy between the results obtained in the two studies may be tentatively explained in a number of ways (see Siesjö 1964) and one possible explanation considers an active transport of hydrogen ions, or of bicarbonate ions across the blood brain barrier.

Thus, if carbon dioxide is administered, a new mean tissue CO_2 tension will be reached in less than 20 min indicating that at that time the tissue concentration of dissolved CO_2, and of carbonic acid will be reasonably constant in all tissue compartments This means that any further increase in the total CO_2 content of the tissue must imply that bicarbonate ions are transferred into the tissue, passively or actively, or that hydrogen ions are carried the opposite way Experiments have shown that there are no significant 'passive' fluxes of these ions between the brain and the blood plasma (Siesjo 1964 b), but it remains to be conclusively shown that the responsible process is an active transport activated by the acidogenic load, and not any one of a number of other hypothetical processes which can explain the disappearance of hydrogen ions from a metabolizing system

Apart from their implication for the study of active transport mechanisms the present results have a bearing also on the elucidation of carbon dioxide transients A quantitative interpretation of such transients in terms of rate limiting factors and compartments would require more experimental data than provided here However, it is tempting to assume that the fast and slow components in the present curves (Fig 2 and 3) are dominated by the lung washout and the tissue blood respectively, and that the faster equilibrium in cortical tissue is due to the higher blood flow (see Kety 1950)

The authors are indebted to the A R C Institute of Animal Physiology Babraham Cambridge and to Dr R D Keynes F R S for providing the facilities necessery to carry the present work out One of us (B K S) wants to thank the Swedish Medical Research Council for a travel grant

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5-Hydroxyindoleacetic Acid of Rabbit Spinal Cord Normally and after Transection

By

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Abstract

Andén, N. E., T. Magnusson, B. E. Roos and B. Werdinius *5-Hydroxyindoleacetic acid of rabbit spinal cord normally and after transection* Acta physiol scand 1965 *64* 193—196 — The normal spinal cord of rabbits was found to contain 0.20 μg/g 5-hydroxyindoleacetic acid in the part cranially to Th4—Th6. Caudally the concentration was 71 per cent of that cranially. After [illegible]

Recent biochemical and histochemical investigations have shown that the 5-hydroxytryptamine (5 HT) in the spinal cord is localized in descending nerve tracts (Carlsson Magnusson and Rosengren 1963 Carlsson *et al* 1964) The 5-HT is highly concentrated in varicosities of axon terminals (Carlsson *et al* 1964) belonging to neurons the cell bodies of which are situated in the lower brain stem (Dahlström and Fuxe 1964 b) Electrical stimulation of these descending pathways results in release and increased synthesis of 5 HT Andén *et al* 1964 a) Administration of the 5 HT precursor 5-hydroxytryptophan produces profound functional changes in the spinal cord (Carlsson, Magnusson and Rosengren 1963 Andén, Jukes and Lundberg 1964, Andén *et al* [illegible]

[illegible] serves as a humoral transmitter there All these investigations have prompted the present study on the effect of transection on the spinal cord levels of 5-hydroxyindole acetic acid (5-HIAA) the main metabolite of 5-HT

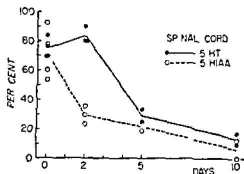


Fig 1 The levels of 5 hydroxyindoleacetic acid (5 HIAA) and 5-hydroxytryptamine (5-HT) of the caudal part of the rabbit spinal cord in per cent of those of the cranial part at various intervals after transection Each symbol represents one single determination

Material and methods

Adult rabbits of both sexes weighing 1 5—3 0 kg were used Under pentobarbital sodium anesthesia (about 30 mg/kg i v) the spinal cord was transected at Th4—Th6 Postoperatively care was taken to prevent hypothermia, residual urine, decubitus and wound infections After various intervals of time the animals were sacrificed by air embolism The spinal cords were [illegible]

concentrated by means of an organic solvent extraction procedure and determined spectrophotofluorimetrically in 3 N HCl (Roos 1962, Roos and Werdinius 1962) The fluorescence spectra were always recorded The recoveries of 2 μg 5 HT and 2 μg 5-HIAA to an aliquot of a homogenate were found to be about 70 and 60 per cent respectively, when no allowance for partition was made All values were corrected for the recovery Normal values were obtained from rabbits not operated upon

Results

Cranially to the transection site the spinal cords of the non operated rabbits were found to contain on the average 0 33 μg/g 5-HT (S E = 0 011, 4 expts) and 0 20 μg/g 5-HIAA (S E 0 031, 4 expts) The levels of these compounds in the cranial part of the spinal cord after the operation were for 5-HT 0 31 μg/g (S E = 0 019, 7 expts) and for 5-HIAA 0 22 μg/g (S E = 0 021, 7 expts) Apparently the transection did not change the concentrations of these substances significantly in the cranial portion of the spinal cord, when analyzed as a whole For this reason it was possible to give the levels of 5-HT and 5-HIAA in the part caudally to the lesion as the percentage of those occurring cranially (Fig 1)

Caudally to the transection site of the non-operated rabbits the concentrations of 5-HT and 5-HIAA were on the average 75 (S E — 3 5, 4 expts) and 71 (S E = 8 8, 4 expts) per cent, respectively of those cranially to it During the first 2 postoperative days the 5-HT in the caudal portion was approximately unchanged It showed a substantial drop between 2 and 5 days and had almost completely disappeared after 10 days Also the 5-HIAA disappeared from the caudal part of the spinal cord after transection The time course of the disappearance of 5-HIAA was however, somewhat different from that of 5 HT In the first 2 days after the operation the 5 HIAA content of the caudal part was reduced from 71 to 29 per cent of that of the cranial one Thereafter there was a gradual drop of the 5-HIAA After 10 days the caudal part was almost completely depleted of 5-HIAA

Discussion

In normal rabbits the proportion between the concentrations of 5-HT and 5 HIAA is about the same in the brain and in the spinal cord The levels are, however, 2—3 times higher in the brain (Roos and Werdinius 1962, Andén, Roos and Werdinius 1963, and unpublished results)

The time courses of the disappearance of monoamines in the spinal cord caudally to a transection have been investigated earlier (Andén *et al* 1964 b) It was found that the depletion of 5-HT is most rapid between the fifth and seventh postoperative day whereas that of noradrenaline is almost finished after 5 days The drop of 5-HT appeared somewhat earlier in the present study which may possibly be due to individual differences It has earlier been observed that the noradrenaline of the rabbit iris disappears at different rates in different animals after excision of the superior cervical ganglion (Andén *et al* 1964 b) However, individual differences cannot be the reason for the finding that the concentration of 5 HIAA in the caudal part was considerably reduced at the second postoperative day when that of 5 HT was about unchanged, since the values were obtained from the same rabbits Probably the level of 5-HIAA better than that of 5 HT mirrors the retardation of the 5-HT metabolism after section of the descending 5 HT pathways in the spinal cord

In the intact spinal cord the 5 HIAA may be formed from the 5-HT released from the nerve granules into the synaptic gap by nerve activity Since this release should cease immediately after section of the descending 5-HT nerves in the spinal cord this effect may be the cause of the rapid disappearance of the 5 HIAA The formation of 5-HIAA can, however probably occur in other ways For example reserpine has been found to produce a long lasting rise of the 5-HIAA in the brain (Ashcroft and Sharman 1962, Roos and Werdinius 1962, Andén, Roos and Werdinius 1963) It can be assumed that reserpine simultaneously reduces the nerve impulse induced release of 5 HT like that of catecholamines from the nerve endings (Carlsson *et al* 1957, Carlsson 1964) by blocking the incorporation of monoamines in the granules (Kirshner 1962 Carlsson, Hillarp and Waldeck 1963, Euler and Lishajko 1963) Therefore the conversion of 5 HT to 5 HIAA can take place intracellularly before the amine is incorporated in the granules The elevated level of 5-HIAA after reserpine treatment may also be due to an enhanced synthesis of 5-HT (Andén, Roos and Werdinius 1963) It has been found that the 5 HT synthesis in the spinal cord is influenced by the activity of the descending nerve tracts (Andén *et al* 1964 a) The disruption of the impulse flow by cutting the spinal cord should, thus cause a diminished synthesis of 5 HT and 5 HIAA This may be responsible for the drop of the 5 HIAA within the first 2 days after the transection although the 5 HT stores are still intact The 5 HIAA remaining after 2 and 5 days may reflect the slow metabolism of 5-HT occurring after denervation It has been observed that the 5 HT metabolism in the caudal part of the spinal cord does not immediately cease after a transection as inhibition of the monoamine oxidase causes a slow accumulation of 5-HT Dahlström and Fuxe 1964 a with the development of 5-HT effects (Andén, Jukes and Lundberg 1964) The approximately simultaneous disappearances of 5-HT and the last of 5-HIAA may occur when the 5 HT terminals are degenerating Anyhow the earlier fall of 5-HIAA after spinal cord transection seems to show that the acid better than the amine reflects the turnover rate of 5 HT

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Distribution of Monoamines and Dihydroxyphenylalanine Decarboxylase Activity in the Spinal Cord

By

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Abstract

Andén, N.-E. *Distribution of monoamines and dihydroxyphenylalanine decarboxylase activity in the spinal cord* Acta physiol scand 1965 64 197—203 — The contents of noradrenaline, 5-hydroxytryptamine and DOPA decarboxylase activity at different levels of the rabbit and cat [illegible] highest in the lateral horns, lower in the anterior and posterior horns and much lower in the white matter The DOPA decarboxylase activity was lower in the lateral horns than in the white matter and the other horns No dopamine was normally found in the spinal cord

The presence of noradrenaline (NA) in the spinal cord was first demonstrated by Euler (1946, 1950) and Vogt (1954) and that of 5-hydroxytryptamine (5-HT) by Amin *et al* (1954) However, until recently nothing was known about the cellular localization and function of these amines in the spinal cord It was possible that they did not occur in central neurons but in vasomotor nerves blood platelets or glia cells Now it has been proved that both amines are localized in descending nerves of the spinal cord (Magnusson and Rosengren 1963, Carlsson, Magnusson and Rosengren 1963, Carlsson *et al* 1964 Andén *et al* 1964 b) and probably serve as synaptic transmitters (Carlsson *et al* 1964, Andén *et al* 1963, 1964 a and c, Andén, Jukes and Lundberg 1964 Andén, Carlsson and Hillarp 1964) These findings prompted studies on the distribution of NA and 5-HT in the spinal cord Such data will be presented and discussed below In addition the activity of L-3,4-dihydroxyphenylalanine (DOPA) decarboxylase was determined in different parts of the spinal cord

Material and methods

Adult cats and rabbits were used for the determination of the concentrations of dopamine (DA), NA and 5-HT at various levels of the spinal cord. The activity of the cord DOPA decarboxylase determined *in vitro*, was also mapped out in these two species The assays of the catecholamines 5-HT and DOPA decarboxylase activity were performed on different animals The cats were sacrificed by bleeding under N_2O anesthesia In the 5-HT experiments the rabbits were anesthetized with pentobarbital sodium (20—30 mg/kg i v) and bled to death from a carotid artery at the same time as they received an infusion of oxygenated Ringer's solution at 37° C into an external jugular vein This procedure eliminated contamination by blood platelet 5 HT In the other experiments the rabbits were sacrificed by air embolism The spinal canal was opened, the dura removed and the spinal cord taken out as soon as possible The spinal roots were used to identify the segments The 1—2 cm of the cord cranial and caudal to the cervical enlargement and the four cranial lumbar segments were removed and discarded in order to permit comparison of representative pieces The pia mater and nerve roots were [illegible]

trophotofluorometrically after ion exchange chromatography and oxidation (Carlsson and [illegible]

in vitro were determined as described above

Results

Distribution of amines in the spinal cord At all levels of the rabbit and cat spinal cord NA and 5-HT were present (Table I) No DA was found normally The distributions of NA and 5-HT were rather even in the rabbit spinal cord The concentrations of both substances were, however, about twice as high in the conus medullaris as in the cranial parts At all levels there was 2—4 times more 5 HT than NA The concentrations of NA and 5 HT in the cat spinal cord were very similar to those found in the rabbits cranial to the lumbar enlargement In the cat lumbar enlargement (L5—S1) the concentrations of both monamines were about twice as high as in the cranial regions The conus medullaris of the cat contained much 5-HT, about five times more than of NA Different animals showed large variations of the concentrations but conspicuously constant distributions There was an almost complete disappearance of both NA and 5-HT in all parts of the spinal cord about 16 hrs after reserpine treatment (cats 5 mg/kg i p, rabbits 2 mg/kg i v)

The concentrations of NA and 5-HT in the anterior and posterior parts of the cat lumbar enlargement were investigated in 2 expts The anterior half was found *to*

TABLE I Distribution of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in the spinal cord of cats and rabbits. The figures in brackets in the headings refer to number of animals. The values are in µg/g tissue and are means with the range in brackets

	Rabbit		Cat	
	NA (2)	5-HT (2)	NA (6)	5-HT (6)
C1—C3	0.09 (0.08—0.10)	0.25 (0.21—0.29)	0.08 (0.04—0.12)	0.18 (0.07—0.28)
Cervical enlargement	0.09 (0.08—0.10)	0.34 (0.33—0.35)	0.12 (0.07—0.14)	0.37 (0.24—0.47)
Th3—L1	0.07 (0.06—0.07)	0.22 (0.21—0.23)	0.11 (0.09—0.13)	0.25 (0.15—0.37)
Lumbar enlargement	0.14 (0.10—0.17)	0.34 (0.34—0.34)	0.23 (0.13—0.34)	0.74 (0.34—1.12)
Conus medullaris	0.26 (0.23—0.29)	0.59 (0.33—0.84)	0.28 (0.17—0.43)	1.41 (0.75—1.93)

TABLE II Distribution of DOPA decarboxylase activity in the spinal cord of cats and rabbits. The figures in brackets in the headings refer to number of animals. The values are expressed in µg dopamine per g tissue formed in vitro after 45 min incubation with L-DOPA or in vivo after injection of L-DOPA. The values are means with the range in brackets

	Rabbit	Cat	
	In vitro (3)	In vitro (2)	In vivo (6)
C1—C3	40 (37—45)	26 (24—27)	1.9 (1.3—3.4)
Cervical enlargement	40 (31—47)	24 (20—27)	2.2 (1.3—4.3)
Th3—L1	33 (31—43)	2[illegible] (23—30)	2.9 (1.0—3.4)
Lumbar enlargement	41 (35—50)	43 (39—47)	2.8 (1.6—4.7)
Conus medullaris	54 (36—69)	77 (66—87)	8.0 (4.5—12.5)

TABLE III *Distribution of noradrenaline* (NA), *5-hydroxytryptamine* (5-HT) *and* DOPA *decarboxylase activity in the thoracic cord of cows* The figures in brackets in the headings refer to number of determinations The values of NA and 5-HT are in µg/g tissue and the DOPA decarboxylase activity is expressed as the formation of dopamine in µg/g tissue after 45 min incubation with L-DOPA The values are means with the range in brackets

	NA (2)	5-HT (2)	*DOPA decarboxylase activity* (2)
Lateral horn	0.23 (0.14–0.32)	0.50 (0.43–0.56)	83 (65–100)
Anterior horn	0.05 (0.05–0.05)	0.14 (0.12–0.16)	350 (330–360)
Posterior horn	0.11 (0.08–0.13)	0.14 (0.14–0.14)	220 (200–230)
White matter	0.01[1]	0.02[1]	270 (240–300)
Grey + white matter before dissection	0.07[1]	0.08[1]	220 (200–240)
after dissection	0.02[1]	0.04[1]	2[illegible]0 (250–2[illegible]0)

[1] Single determination

contain 0.12 µg/g NA and 0.66 µg/g 5-HT whereas the corresponding values of the posterior half were 0.14 µg/g and 0.67 µg/g, respectively

Distribution of DOPA decarboxylase activity in the spinal cord The distribution of DOPA decarboxylase activity in the spinal cord of rabbits and cats (Table II) was very similar to those of the monoamines In the rabbit cord the activity, when determined *in vitro*, was rather evenly distributed but was somewhat higher in the conus medullaris than in the other regions In the cat spinal cord the *in vitro* activities of the parts cranial to the lumbar enlargement were somewhat lower than those of the corresponding parts in rabbits The lumbar enlargement contained more than the cranial portions and the conus medullaris still more in agreement with the higher monoamine concentrations in these regions Since no DA was found normally in the cat spinal cord and the NA formation was negligible, the DA concentration after injection of L-DOPA should reflect the enzyme activity *in vivo* As seen in Table II the activities obtained *in vitro* and *in vivo* agreed satisfactorily

Distribution of amines and DOPA decarboxylase activity in the thoracic cord The distributions of NA and 5-HT in the cow thoracic cord are presented in Table III Like in the cat and rabbit spinal cords no DA was observed The highest concentrations of NA and 5-HT occurred in the lateral horns These substances were present also in the

anterior and posterior horns but in lower concentrations. The posterior horns included also the intermedio-medial nucleus and the substantia grisea centralis (Rexed 1954). In the white matter the two amines occurred in very low concentrations. In all places there was more 5-HT than NA.

The distribution of the DOPA decarboxylase activity, determined *in vitro*, was apparently different from those of the amines in the thoracic cord (Table III). The enzyme activity was high in the white matter as well as in the anterior and posterior horns whereas it was much lower in the lateral horns.

Discussion

In all locations of the brain where 5 HT occurs there is also a catecholamine, usually NA but DA in *e g* the caudate nucleus and the putamen (Bertler 1961 b). The same holds true for the spinal cord with NA as the only catecholamine found normally. The NA and 5 HT in the spinal cord are, however, much more evenly distributed than in the brain. The proportion between the two amines is also strikingly constant at the different levels of the spinal cord. In fact the small differences between the concentrations of NA and 5 HT in the various parts of the rabbit spinal cord may be explained by similar differences in the amount of grey matter (calculated from the illustrations in Craigie 1949 p 77). In the cat the differences in concentrations of NA and 5 HT between the enlargements and the thoracic cord may be accounted for by a similar distribution of the grey matter (calculated from illustrations in Rexed 1954). However, the cranial cervical segments of the cat cord have lower concentrations of both NA and 5 HT than the other parts even if allowance is taken for the somewhat smaller content of grey matter. In the conus medullaris there is a very high concentration of 5 HT, actually the highest found in the central nervous system of cats in this laboratory. The hypothalamus, which has the second highest 5 HT concentration, contains about 0 7 $\mu g/g$. The cellular localization of the 5 HT in the cat sacral region has been studied by the histochemical fluorescence method (Dahlström and Fuxe unpublished data). In the anterior horns there are abundant 5-HT terminals, especially in the ventral and ventrolateral parts and around the central canal. In the superficial zone of the posterior horns there are numerous descending 5-HT axons the terminals of which probably are submicroscopic. Further there are many 5-HT terminals in the twisted parasympathetic nucleus (Rexed 1954) but not as many as in the sympathetic column of the thoracolumbar cord.

From the results on the distribution of DOPA decarboxylase activity in the rabbit and cat spinal cord [illegible]

amines is localized in descending nerves of the spinal cord (Andén, Magnusson and Rosengren 1964 a and b) and that it probably catalyzes the biosynthesis of both catecholamines and 5 HT (Westermann *et al* 1958, Yuwiler *et al* 1959, Bertler and Rosengren 1959 b, Rosengren 1960).

The results from the few investigations of the distribution of amines and DOPA decarboxylase activity in the thoracic cord must be looked upon as preliminary, espe- [illegible]

that both monoamines have their largest accumulation in the lateral horns and are almost

completely lacking in the white matter. These findings are in agreement with the observations made by the histochemical technique (Carlsson *et al.* 1964). Because of the small weight of the lateral horns their monoamines do not contribute by more than at most 10 per cent of the total monoamine content of the thoracic cord. This fact probably explains why the monoamine concentrations of the thoracic cord are not larger than those of the other cord parts in the rabbit and cat experiments. The discrepancy between the distribution of the amines and the DOPA decarboxylase activity in the cow thoracic cord as well as the high decarboxylase activity in the whole thoracic cord of this species are obscure. The histochemical method has shown that the monoaminergic nerves in the spinal cord begin to display varicosities with high amounts of monoamines prior to their entrance in the grey substance where all synaptic contact appears to take place (Carlsson *et al.* 1964). Part of the DOPA decarboxylase in the white matter may be localized in such varicosities. Also the non terminal parts, however, of the monoaminergic neurons contain this enzyme (Holtz and Westermann 1956, Andén, Magnusson and Rosengren 1964 b). It may be conceivable that the descending monoaminergic axons occupy a larger part of the white matter of the thoracic cord of cows than of the smaller animals. If the relatively low DOPA decarboxylase activity found in the lateral horns is not due to technical errors it may possibly indicate a lower synthesis rate in the nerves belonging to the central part of the autonomic nervous system than in those belonging to other systems. The proportion between DOPA decarboxylase activity and amine concentration in the lateral horns is similar to that found in sympathetically innervated organs (Andén, Magnusson and Rosengren 1964 b) but lower than that found in other parts of the central nervous system (Bertler and Rosengren 1959, unpublished data).

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The Metabolism of Fatty Acids in the Rat

VII. Linoleic Acid

By

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Abstract

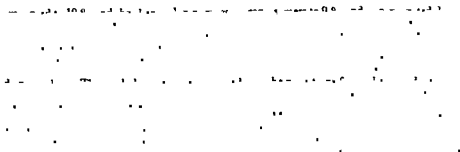
recovered from the fasted rats The distribution of radioactivity between the neutral lipid and phospholipid fractions in the separate tissues from the fasted rats agreed with the fatty acid composition of each fraction The pattern of radioactivity in the blood phospholipids at 320 min closely resembled that in the liver phospholipids at 5 min

Previous investigations in our laboratory have been concerned with the metabolism in the rat of six different fatty acids (Goransson and Olivecrona 1964 and 1965, Goransson 1964 a, b, c, d)

The present article reports the results from experiments with linoleic acid The purpose was to study if this essential fatty acid is metabolized differently compared to the non essential palmitic acid in normal rats The two acids, labeled with C^{14} and H^{3} respectively were therefore injected together into rats of different nutritional state and the following parameters studied disappearance of label from the blood, uptake of label into the tissues, oxidation of label, incorporation of label into tissue lipids and recirculation of label back into the blood

Methods

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cis-cis-methyl linoleate and the origin

The methyl esters were hydrolyzed with 4 % KOH in 95 % ethyl alcohol in water at room temperature overnight The fatty acid was finally extracted with petroleum ether from an

An injection solution of the two acids in rat serum was prepared as described earlier (Goransson and Olivecrona 1964 and 1965) The injected dose of 0 5 ml of serum contained approximately 0 03 micro-Eq of both the labeled linoleic acid and the labeled palmitic acid

Results

In the present experiments the behaviour of the labeled palmitic acid agreed with the results of earlier work (Goransson and Olivecrona 1964) Therefore in this article the the results are given as the ratio of linoleic acid label (C^{14})/palmitic acid label (H^3) The ratio in the injected fatty acids was taken as one

The results can be seen in Tables I—IV

Table I shows the disappearance of label from the blood Linoleic acid was extracted from the circulating blood at a higher rate than palmitic acid in both fasted and refed rats The refed rats showed the greatest difference

Table II gives the ratios between C^{14}-label and H^3-label in blood lipid fractions at 40 and 320 min These time intervals after injection were chosen because earlier work (Goransson and Olivecrona 1964, Laurell 1959) has shown that the recirculation of labeled fatty acid in the triglyceride fraction of blood or plasma reaches a maximum around 40 min Furthermore, considerable amounts of label do not appear in the blood phospholipid and cholesterol ester fractions until several hours after the injection of the labeled fatty acids The present results indicate that at 320 min linoleic acid label had been incorporated to a larger extent into the blood cholesterol esters than palmitic acid label both in the fasted and the refed rats At 40 min the blood glycerides of the fasted rats contained less linoleic acid label than palmitic acid label, whereas the reverse was true in the refed rats In the phospholipids from both fasted and refed rats the ratio of linoleic acid label to palmitic acid label was smaller than unity at 320 min

In Table III the radioactivity of the total lipid extracts from different tissues and from the whole rats can be seen The labeled linoleic acid was more rapidly oxidized than the palmitic acid label in the fasted rats but in the refed rats the ratio between linoleic and palmitic acid label was rather close to unity The same tendency was found in the separate tissues In the fasted rats the ratio was well below one in all the tissues

TABLE I C^{14}/H^{3} radioactivity in the blood FFA fraction in rats after the i v injection of C^{14} linoleic and H^{3} palmitic acid in rat serum Each value is the mean ± SEM of 3 rats The ratio in the injected fatty acids was taken as 1 0

Min	Fasted rats	Refed rats
1	0 68±0 04	0 58±0 02
2	0 50±0 05	0 31±0 04
3	0 47±0	0 29±0
4	0 43±0 06	0 24±0 04
5	0 44±0 03	0 19±0 03

TABLE II Ratio C^{14}/H^{3} radioactivity in the blood lipids in rats after the i v injection of C^{14} linoleic and H^{3} palmitic acid in rat serum Each value is the mean ± SEM of 3 rats The ratio in the injected fatty acids was taken as 1 0

	Fasted rats			Refed rats		
Min	Cholesterol esters	Glycerides	Phospholipids	Cholesterol esters	Glycerides	Phospholipids
40	0 86±0 12	0 80±0 09	0 [illegible]3±0	1 43±0 20	1 13±0 03	0 81±0 04
320	1 97±0 21	1 17±0 08	0 69±0 02	1 48±0 13	1 05±0 05	0 95±0 09

TABLE III Ratio C^{14}/H^{3} radioactivity in the total tissue lipids from rats after the i v injection The ratio in the injected fatty acids was taken as 1 0 'Total in the rat' represents

Min	Liver	Adipose tissue	Muscle	Heart
	Fasted rats			
5	0 84±0 02	0 83±0 02	0 89±0	0 72±0 09
320	0 69 0 02	0 62±0 22	1 00±0 05	0 73±0 01
	Refed rats			
5	0 97±0 01	0 82±0 01	0 85±0 01	1 07±0 02
320	0 87±0 02	1 04±0 03	0 87±0 01	1.54±0 15

TABLE IV Ratio C^{14}/H^{3} radioactivity in the neutral lipid and the phospholipid fractions of tissue lipids from fasted and refed rats after the i v injection of C^{14} linoleic and H^{3} palmitic acid in rat serum. The values represent the mean ± SEM of 3 rats The ratio in the injected fatty acids was taken as 1 0

Min		Liver	Muscle	Kidneys	Lungs	Spleen
	Fasted rats					
5	Neutral lipids +FFA	1.02 ± 0.03	0.83 ± 0.01	0.62 ± 0.07	0.54 ± 0.01	0.35 ± 0.01
	Phospholipids	0.71 ± 0.01	1.10 ± 0.06	0.58 ± 0.05	0.44 ± 0.01	0.62 ± 0.03
320	Neutral lipids +FFA	0.93 ± 0.04	0.99 ± 0.05	0.57 ± 0.01	1.21 ± 0.14	0.43 ± 0.09
	Phospholipids	0.62 ± 0.02	0.99 ± 0.08	0.60 ± 0.06	0.35 ± 0.04	0.46 ± 0.01
	Refed rats					
5	Neutral lipids +FFA	0.99 ± 0.02	0.54 ± 0.02	0.58 ± 0.01	0.52 ± 0.01	0.39 ± 0.02
	Phospholipids	1.06 ± 0.02	1.31 ± 0.06	1.03 ± 0.03	0.90 ± 0.03	0.82 ± 0.01
320	Neutral lipids +FFA	0.97 ± 0.01	0.56 ± 0.03	0.76 ± 0.02	0.81 ± 0.01	0.85 ± 0.14
	Phospholipids	0.87 ± 0.04	1.09 ± 0.01	0.91 ± 0.02	0.74 ± 0.02	0.65 ± 0.01

of C^{14} linoleic and H^{3} palmitic acid in rat serum Each value is the mean ± SEM of 3 rats the ratio in the sum of the radioactivity in all the tissues taken out plus that in the carcass

	Kidneys	Lungs	Spleen	Carcass	Total in the rat
	0.59 ± 0.05	0.43 ± 0.01	0.51 ± 0.01	0.87 ± 0.01	0.84 ± 0.01
	0.58 ± 0.03	0.36 ± 0.01	0.42 ± 0.01	0.95 ± 0.04	0.86 ± 0.03
	0.89 ± 0.02	0.64 ± 0.01	0.65 ± 0.01	0.97 ± 0.04	0.95 ± 0.03
	0.90 − 0.02	0.65 ± 0.02	0.60 ± 0.01	1.00 ± 0.01	0.97 ± 0.01

and in the refed rats the ratio was higher in all tissues than in the fasted rats In the livers from both fasted and refed rats the ratio at 320 min was smaller than that at 5 min

Table IV, finally, shows the relative incorporation of linoleic acid label to that of palmitic acid label into the neutral fat + FFA and the phospholipids In some tissues from fasted rats more linoleic acid label than palmitic acid label was incorporated into the phospholipids as compared to the glycerides after 5 min and in other tissues the reverse was true In the refed rats, however, there was always more linoleic acid label in the phospholipids at 5 min than in the glycerides

Discussion

Earlier experiments by Mead *et al* (1956) and by Blomstrand (1954) showed that when fed in excess of amounts needed linoleate was oxidized more rapidly than fed stearate and palmitate in the rat Lossow and Chaikoff (1959) found that the oxidation of linoleic and linolenic acid was spared by glucose Thus in these investigations no specific sparing of the essential fatty acids was noted The same conclusion can be drawn from the present experiments, in which the injected linoleic and palmitic acids were metabolized by the same principal pathways

Cis cis linoleic acid 1 C^{14} was found to disappear from the blood at a considerably higher rate than the H^3 palmitic acid both in fasted and refed rats In experiments carried out on humans Fredrickson and Gordon (1958) originally did not observe any difference in the extraction rate of i v injected linoleic and palmitic acid Later however Dustin *et al* (1961) injected H^3 palmitic acid and 1 C^{14} linoleic acid together into rats and noted that the C^{14} label disappeared more rapidly than the H^3 label from the blood These findings together with the results presented in this work make it probable that in the rat linoleic acid is in fact extracted at a higher rate from the blood than palmitic acid

At 5 min after injection practically all the labeled linoleic and palmitic acid had disappeared from the blood in the refed rats Therefore the total tissue radioactivity in the refed rats at 5 min after the injection most likely reflects the initial distribution of label since at that time little oxidation or redistribution of label between separate organs has taken place (Goransson and Olivecrona 1964) A ratio of linoleic acid label to palmitic acid label greater than unity was only found in the heart This might not have been expected in view of the results of Carlsten *et al* (1963 who showed that the a v difference over the human heart for linoleic acid was smaller than that of all other fatty acids studied In the lungs and the spleen the ratio C^{14}/H^3 suggested a preferential uptake of palmitic acid

Once the labeled fatty acids have been taken up by the cells they are directly available for oxidation interconversion and incorporation into separate lipid fractions followed by recirculation back into the blood as fatty acid esters The different pathways will be discussed in some detail below

Oxidation

In the fasted rats less linoleic acid label than palmitic acid label was recovered from the whole rat 5 min after injection It should be recalled at this point however that the linoleic acid label disappeared more rapidly from the blood than the palmitic acid

label Thus linoleic acid and palmitic acid may still be equally available for oxidation immediately after the entry into the cell, in spite of the low ratio obtained in the present experiments After 320 min the amount of the two isotopes had declined parallelly so that the ratio was still the same This is partly explained by the fairly equal incorporation of the two isotopes into neutral lipids and phospholipids

The finding in this work that linoleic acid was more rapidly oxidized than palmitic acid agrees with earlier results (Mead *et al* 1956 and Blomstrand 1954) that indicated a more rapid oxidation of fed linoleic acid than palmitic acid in rats.

Interconversion

Theoretically linoleic acid might be oxidized to 2-carbon units, which could then give rise to fatty acids other than linoleic In the present experiments, however this indirect interconversion does probably not take place to any large extent, because in the refed rats the overall oxidation is very small and in the fasted rats the synthesis of fatty acids from 2-carbon units is depressed

Another way of interconversion would be hydrogenation, but this reaction is considered to be of minor importance in higher animals (Mead 1960)

The conversion of linoleic acid to arachidonic acid constitutes a third possible way of interconversion That this reaction does indeed take place was shown by Mead (1953) However, in the experiments of Dittmer and Hanahan (1959) labeled arachidonic acid was not found to any large extent except in phosphatidyl-ethanolamine (26 %)

The interconversions of palmitic acid have been discussed elsewhere (Elovson 1965)

Incorporation of radioactivity into neutral lipids and phospholipids

In the fasted rats where no interconversion of palmitic acid takes place, it was found that incorporation of linoleic acid label as compared with palmitic acid label took place according to the fatty acid composition in the respective lipid fraction found earlier (Goransson and Olivecrona 1964) except in the spleen

Recirculation back into the blood

As could be expected from earlier work Goransson 1964 a and b the pattern of label in the blood phospholipids at 320 min was similar to that in the liver phospholipids at 5 min The ratio in the blood glycerides did not resemble the ratio in the liver neutral lipid fraction as closely as in earlier work with other fatty acids

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Direct Studies on the Disappearance of the Transmitter and Changes in the Uptake-Storage Mechanisms of Degenerating Adrenergic Nerves

By

TORBJORN MALMFORS and CHARLOTTE SACHS

Received 11 October 1964

Abstract

Malmfors, T and Ch Sachs *Direct studies on the disappearance of the transmitter* [illegible] *e uptake-storage mechanisms of degenerating adrenergic nerves* [illegible]
— The histochemical fluorescence [illegible]
the disappearance of th [illegible] usins
of the adrenergic nerves [illegible] ation following axotomy
It was found that the tra [illegible] terminals does not disappear gradually but quite suddenly and with various times of onset for the different systems of terminals The uptake and accumulation of NA and α methyl-NA were examined in untreated and reserpinized animals. These experiments show that the uptake-storage mechanisms in any given system of terminals operate up to a certain time without any obvious changes, but then deteriorate rapidly, at about the same time as the transmitter stores disappear The axon membrane reabsorption mechanism seems to be lost somewhat prior to the storage mechanism of the transm [illegible]
same time as degenerative changes seem to occur in the [illegible]
a passive leakage of NA — The [illegible]
component in the denervation [illegible]
— Following the administratio [illegible] were delayed for about 8 hrs

The disappearance of the transmitter and the degenerative changes occurring in the adrenergic nerves following axotomy have recently been studied with renewed interest (see *e g* Kirpekar *et al* 1962, Trendelenburg 1963 a and b Benmiloud and Euler 1963 Fleming 1963 These studies have concerned themselves especially with current hypotheses as to the cause of denervation supersensitivity and it has been proposed that loss of the mechanisms for amine uptake is to a great extent responsible for such supersensitivity as it permits higher concentrations of catecholamines (CA) to reach the adrenergic receptors

The adrenergic nerves and their transmitter can now be studied at cellular and subcellular levels in an entirely new and direct manner using the highly sensitive fluorescence method of Falck and Hillarp for the histochemical demonstration of certain monoamines. As shown previously (Malmfors 1965 a), this can be done especially successfully on whole mounts of albino rat iris which permit a close examination of the adrenergic axons and terminals throughout the tissue with respect to the occurrence, distribution, uptake and storage of the transmitter.

In the present work, the rat iris technique has been used *for studies of the changes* occurring in the nerves during the course of degeneration following axotomy, with respect to the intraneuronal distribution and disappearance of the transmitter, the loss of amine uptake-storage mechanisms and the effects of reserpine, bretylium and a potent monoamine oxidase (MAO) inhibitor (nialamide).

Material and methods

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Results

1 Changes in content and distribution of the adrenergic transm tter during the course of degenerat n following

... NA and also of DA DOPA and their ... which exh bit an intense fluorescence ... Falck *et al* 1962 Corrodi and Hillarp ... f the method see Norberg and Hamberger 1964) the adrenerg ... ostrated and its intraneuronal distribution and the adrenergic innervation apparatus can thus be stud ed

Fig. 1. Iris of normal rat. In addition to the strongly fluorescent and uniformly distributed plexus of adrenergic nerve terminals over the dilator muscle and around an arteriole, there is seen a nerve trunk with weakly fluorescent non varicose main axons (⟶). × 160



Fig. 2. Rat iris 16 h after axotomy. The main part of the nerve plexus has disappeared, leaving some branching terminals of normal appearance (type N). No fibres of type D are present. Some preterminal fibres are seen (⟶). × 160

than in operated animals not pretreated with nialamide. Non-terminal axons are also seen (⟶) × 250

The adrenergic innervation of the iris has been described in detail in other papers (Malmfors 1965 a and b, Malmfors and Sachs 1965) The sympathetic fibres arrive at the periphery of the iris These main axons continue in small bundles (Fig 1) and finally give off several thin smooth branches, preterminal axons (Fig 2) Each of the preterminal axons ends with a system of branching terminals (Fig 4) which have abundant enlargements (varicosities The varicosities — in contrast to ...
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... autonomic ground plexus (see Hillarp 1959) and that two or more systems of terminals often converge to the same effector cells — Further microphotos of normal and degenerating iris nerves are to be found in Malmfors (1965a)

Irises from about 70 animals were examined from 8 to 40 hrs after axotomy No obvious changes in the appearance of the adrenergic nerves were found at 8 hrs After 28 hrs or more, the stored transmitter had either completely disappeared or else — in about 10 per cent of cases — there were left a few systems of branching and apparently intact terminals no doubt originating from 1 or 2 sympathetic ganglion cells located peripherally to the axotomy (see Malmfors and Sachs 1965)

A closer examination of the degeneration changes revealed the following

a) The transmitter stored in the terminals did not disappear gradually from the entire innervation apparatus at once System after system of branching terminals distributed more or less randomly throughout the iris (Fig 2), lost their NA content completely, while — at any given point of time after axotomy — most of the remaining terminals appeared normal (type N) and the rest had only weakly fluorescent varicosities and exhibited no only or a very weak fluorescence in the segments between these (type D) Rough estimates of the numbers of these two types of terminals remaining at different degeneration times are found in Table 1 The figures for 12 hrs are probably too high, since it is difficult to estimate the magnitude of a disappearance when most of the terminals still remain There is little doubt however that a large proportion of the terminals were affected and lost their NA during the four hours between 12 and 16 hrs after axotomy This and the finding that no clear transitional stages seemed to exist between the type N and type D terminals show that the terminals at some point of time — which differs from system to system — lose their transmitter content quite suddenly (probably within 1 or at the most 2 hrs) The same findings also seem to indicate that degeneration proceeds in 2 phases first a rapid disappearance of most of the stored amine and then a somewhat slower disappearance of the remaining contents

b) The branching terminals belonging to the system arising from a single preterminal fibre reacted uniformly along their entire lengths during the course of degeneration This could be observed especially at 16 and 20 hrs after axotomy when many or most of the terminals had disappeared completely The remaining systems of terminals of both types were distributed more or less randomly throughout the iris The same findings were made in the experiments with nialamide treatment and amine uptake described below

c) The main postganglionic axons and the preterminal fibres seemed to lose their low NA content at approximately the same time after axotomy as the terminals Numer-

ous non terminal axons were seen and their course and branching could be easily studied at 16 and 20 hrs (Fig 2, 3) None or very few remained at 24 hrs. The earliest sign of degeneration was the appearance of usually small enlargements irregularly distributed along the fibres

Differences in the time course of the degeneration were observed between the two irises from the same animal, but were greater from animal to animal at the same degeneration time

2 *Reserpine induced depletion of the adrenergic transmitter during degeneration*

A large dose of reserpine (10 mg/kg) was administered to 3 groups of 4 rats immediately after cervical sympathectomy and the animals examined after 2, 4, and 8 hrs Just as in animals with intact innervation (see Malmfors 1965 a), the fluorescence was strongly reduced after 2 hrs while very few and weakly fluorescent terminals remained at 4 hrs and all fluorescent nerves had disappeared after 8 hrs.

3 *Effects of nialamide treatment during degeneration*

The adrenergic nerves were examined 4 hrs after a single large dose of nialamide (500 mg/kg 42 rats, degeneration times from 12 to 36 hrs) or 4 hrs after the last of 2 to 6 doses 100 mg/kg 18 rats, degeneration times from 16 to 24 hrs) administered at intervals of 4 hrs

The administration of this potent MAO inhibitor did not result in any obvious delay in the disappearance of the transmitter Table I The terminals of type N remaining at the various times showed a normal appearance but a distinctly increased fluorescence intensity (A generally increased transmitter content has also been found in the terminals of animals with intact innervation Malmfors 1965 a No type D terminals with weakly fluorescent varicosities were observed but there were found instead terminals similar to type D terminals in respect of their occurrence at 16 and 20 hrs and the weak to non-existent fluorescence of the segments between the varicosities The latter however were more pronounced and exhibited a strong fluorescence Fig 4 There seems to be little doubt that these terminals are identical with the type D terminals see Discussion — The non terminal axons showed an increased fluorescence intensity (Fig 4

4 *Amine uptake mechanisms during degeneration*

It has been shown in previous papers (Hamberger *et al* 1964 Hillarp and Malmfors 1964 Malmfors 1965 a that the entire postganglionic axon possesses a mechanism for the uptake-concentration of NA and related amines which is localized to the level of the axon membrane This mechanism can be studied after the administration of a large dose of reserpine which causes a complete depletion of the endogenous NA and blocks the storage function of the granules Carlsson 1965)

It is possible directly to demonstrate the uptake of NA or *α* methyl NA following the administration of very low doses of the amines i v or intraocularly In the present experiments however it was found valuable to give high doses 1 and sometimes up to 10 mg/kg i p in order to obtain a very marked accumulation of the amines 1 hr after their administration In most of the experiments 46 rats *α* methyl NA was given alone and NA 4 to 5 hrs after the administration of nialamide 500 mg/kg since NA — but not *α*-methyl NA — breaks down very rapidly intraaxonally if the granule mechanism is not intact or MAO not efficiently inhibited *cf* Malmfors 1965a

TABLE I Rough estimates per cent of remaining adrenergic terminals of normal appearance (*N*) or with weak fluorescence (*D*)

Degeneration time Hrs	Treatment of the animals: Untreated	Nialamide	Nialamide + NA (or α-methyl NA)	Reserpine + nialamide + NA (or α-methyl NA)	Bretylium tosylate
8	100 (N)			100 (N)	
12	92—100 (N)	95—100 (N)	95—100 (N)	95—100 (N)	
16	25— 40 (N)	25— 50 (N)	25— 45 (N)	30— 50 (N)	
	10— 20 (D)	10— 15 (D)	10— 20 (D)*		
20	5— 15 (N)	10— 15 (N)	10— 20 (N)	5— 10 (N)	90— 95 (N)
	2— 5 (D)	1— 5 (D)	1— 5 (D)*		
24	0— 2 (N)	1— 3 (N)	1— 4 (N)	0— 1 (N)	25— 45 (N)
					5— 10 (D)
28	0— 1 (N)	0— 1 (N)	0— 1 (N)	0	

Disappearance of the transmitter in the adrenergic terminals of the rat iris after cervical sympathectomy.—The rats — usually 5 to 7 animals in each group — were either untreated or treated with one or several doses of nialamide or bretylium tosylate. The amine uptake mechanisms of the degenerating terminals were studied in rats killed 1 hr after a large dose of NA (pretreatment with nialamide) or α-methyl NA. In one of these experiments, the endogenous NA was first depleted by administration of a large dose of reserpine 1 hr after the operation.—The terminals of type D were degenerating and had lost most of their transmitter (weakly fluorescent varicosities). After nialamide treatment or the administration of NA, however, the *corresponding terminals* (marked with an asterisk*) showed *strongly* fluorescent varicosities (see Sections 3 and 4).

The same results were obtained in both cases. The effects of degeneration on uptake were studied at 12 to 40 hrs after axotomy, but most of the experiments were made at the critical times of 16 and 20 hrs.

No accumulation could be obtained after degeneration times of 24 hrs or longer. Large doses of DA (5 to 100 mg/kg i. p.) or L-DOPA (20 to 100 mg/kg i. p.) to animals (30) pretreated with nialamide were also ineffective.

The amines accumulated in the adrenergic nerves in normal or reserpinized animals remain for many hours, provided that MAO activity is efficiently inhibited (see Malmfors 1965 a). However, no obvious delay was observed (Table I) in the disappearance of the two types of terminals, in spite of the fact that high accumulations similar to *those obtained in intact nerves found in the degenerating nerves at 12 hrs* — when all the terminals seemed to remain — and in the terminals remaining at 16 and 20 hrs. The type N terminals exhibited a very strong fluorescence and the increase — just as in normal terminals — was most marked in the segments between the varicosities, which gave the fibres a more smooth appearance. No weakly fluorescent type D terminals

could be observed, but terminals which no doubt correspond to this type had varicosities exhibiting a very strong fluorescence and seemed also to be larger (this may be a degenerative swelling, which can be clearly seen only after a high accumulation of amines) In marked contrast to intact terminals no or only a very weak fluorescence could be obtained in the segments between the varicosities (Fig 3) — No increase in fluorescence was observed 30 min after the administration of NA alone

Many non terminal axons showed a high amine accumulation 16 and 20 hrs after axotomy, their course and branching could be readily followed in the entire iris and their rate of disappearance could also be successfully studied (Fig 3) In agreement with the findings reported in the previous section, the fluorescence of these axons seemed even after a massive accumulation of amines to disappear at about the same time as the terminals — or perhaps somewhat later — since many strongly fluorescent non terminal axons were observed 20 hrs after axotomy

5 *Amine uptake mechanisms during degeneration in reserpinized animals*

The uptake and accumulation of NA or α methyl NA (for doses and times, see section 4) were examined in 70 rats given reserpine (10 mg/kg) 1 hr and killed 8 to 32 hrs after axotomy Unless otherwise stated, nialamide was administered 4 hrs before NA About the same results were obtained with nialamide plus NA as with α methyl NA alone

No accumulation was found at degeneration times of 24 hrs or longer Terminals showing a high amine accumulation were found at 8 to 20 hrs after axotomy, with a frequency and distribution about the same as those of the type N terminals in untreated animals (Table I) These terminals — just as in reserpinized animals with intact innervation — showed a more "smooth" appearance, with a strong fluorescence also in the segments between the varicosities No terminals were observed, however, that would correspond to type D with strongly fluorescent varicosities but exhibiting no or only weak fluorescence in the segments between

No NA was found to remain 30 min after administration in animals not pretreated with nialamide, unless the rats were killed within 15 min after the NA injection and the irises left *in situ* for 15 min before being taken out This treatment produces anoxia in the iris and thus an inhibition of MAO (see Malmfors 1965 a)

The non terminal axons showed a high amine accumulation and many remained at 20 hrs after axotomy, just as in the non reserpinized animals in the previous series

The disappearance of the NA accumulated 4 to 8 hrs before killing of the animals was examined in 16 rats 12 to 20 hrs after axotomy The strongly fluorescent terminals disappeared at about the same rate as type N in untreated animals The terminals remaining at 12 and 16 hrs showed a reduction in their accumulated amines that was similar to the terminals in reserpinized animals with intact innervation (see Malmfors 1965 a)

6 *Effects of bretylium during degeneration*

Bretylium tosylate was administered in doses of 50 mg/kg at intervals of 4 hrs starting immediately or 8 hrs after cervical sympathectomy The animals (12) were killed 20 or 24 hrs after operation

In contrast to the untreated animals the rats treated with bretylium showed no or only a small decrease in the number of terminals or in their fluorescence intensity 20 hrs

after operation (Table I) Four hours later there were still many strongly fluorescent terminals of apparently normal appearance and there were observed now also type D terminals As seen in Table I, however, the disappearance of the transmitter in the interval between 20 and 24 hrs was about the same as that in the interval between 12 and 16 hrs in the animals not treated with bretylium

The non terminal axons showed about the same fluorescence as in untreated animals They were numerous at 24 hrs, when there was a marked reduction of the terminals

Discussion

The time course of the disappearance of the adrenergic transmitter in the brown adipose tissue (Weiner *et al* 1962) and the submaxillary gland (Benmiloud and Euler 1963) of the rat has been examined during degeneration of the sympathetic nerves The NA content was unchanged at 8 hrs but lost by 24 hrs after axotomy The present results are in complete agreement with these observations Our data on the disappearance' of the adrenergic terminals (Table I), which contain practically the entire transmitter store, agree well with the chemical data on the NA decrease in the submaxillary gland in the interval between 8 and 24 hrs, since — as already pointed out — our 12 hr figures are in all probability too high —The adrenergic nerves in the rabbit iris have also been found to lose their NA gradually in the interval between 8 and 24 hrs after axotomy (Andén *et al* 1965) In other species degeneration seems to be somewhat slower (see Hillarp 1960, Kirpekar *et al* 1962, Wegmann *et al* 1962, Sedvall 1964)

Studies by the methods used hitherto on the disappearance of the transmitter during degeneration of the nerves have given little information as to the mechanisms of this disappearance and their relation to physiological or degenerative processes in the axons Although this has not been directly stated, the results obtained seem generally to have been interpreted on the basis of the reasonable assumption that the rate of disappearance determined reflects by and large the events in the individual adrenergic nerves When the present study was started there was no obvious reason to doubt the validity of this assumption It shows clearly, however that the transmitter does not disappear gradually over a long period from most or even from the greater part of the terminals as might have been thought All the observations show instead that any one of the systems of terminals — each consisting of the branching terminals arising from a single preterminal axon — lose their NA content quite suddenly, but that there are great differences between the systems in the time of onset The gradual decrease in tissue NA is consequently due to system after system of terminals dropping out" more or less randomly throughout the tissue, while the remaining systems mostly seem to be intact with respect to transmitter content but become fewer and fewer Although the terminals studied all belong both anatomically and functionally to the same population, the numerous systems within the population show internally a great biological variation

The terminals remaining at any given time after axotomy did not for the most part (type N) differ from normal terminals with respect to morphology distribution of stored transmitter amine increase after administration of nialamide (*cf* Kopin 1964) or amine uptake and accumulation before or after reserpine (see below) Some of the terminals (type D) showed marked and characteristic changes The abundant va

ricosities that in normal terminals contain practically the entire amount of the NA stored in the adrenergic nerves and which are in all probability specialized structures for the storage and release of the transmitter (see Norberg and Hamberger 1964 Malmfors 1964, 1965 a) had lost most of their amine content but increased in size, which suggests that degenerative swelling had occurred As discussed below, *there is* good evidence that these terminals had also lost one of the mechanisms for amine uptake and concentration Since there is little doubt that the terminals finally lose the remaining transmitter and their property to accumulate amines at about the same time, these findings indicate that the terminals belonging to one system are fairly normal up to a given time after axotomy *but then lose most of their transmitter content quite* suddenly, probably due to degenerative changes which result in swelling and a deter ioration of the uptake-concentration mechanism localized to the level of the axon membrane After this rapid phase, the remaining transmitter is lost somewhat more slowly, probably due to deterioration of the uptake storage mechanism of the amine granules (see below) *The degeneration thus seems to proceed in two phases*

There is strong evidence that the storage granules in the adrenergic nerves isolated and extensively studied by Euler and co-workers (Euler and Hillarp 1956, Euler 1958 Euler and Lishajko 1961, 1963 Stjarne 1964), contain most of the transmitter normally present in the non terminal axons and terminals and that the amines in this way *are protected from inactivation by the MAO existing within the axons themselves* (Hamberger *et al* 1964, Hillarp and Malmfors 1964, Malmfors 1965 a) It could further be directly demonstrated that not only the terminals but the entire postgang lionic axon possess a highly efficient mechanism for the uptake concentration of NA which is localized to — or at least to the level of — the axon membrane and — in contrast to the granules — *is highly insensitive to reserpine There is little doubt that* the mechanism for amine uptake by the granules is effectively blocked by reserpine (see also Carlsson 1965 Dahlstrom *et al*)

The observations on the uptake and accumulation of NA (or α methyl NA) by the terminals and non terminal axons clearly show that there is a very intimate correlation between the disappearance of the endogenous NA and severe changes *in the uptake* storage mechanisms The amines administered were readily taken up and accumulated to very high concentrations in the terminals both before and after a reserpine induced transmitter depletion up to 8 hrs after axotomy In contrast to this no accumulation at all could be obtained in any part of the axons at degeneration times of 24 hrs or longer *The observations made on closer examination of the uptake-storage mechanisms in the* non reserpinized animals strongly support the view that these mechanisms in any given system of terminals operated up to a certain time without any obvious *changes* but then deteriorated rapidly and at about the same time as the transmitter stores disappeared This view received further strong support from the results obtained when the accumulation and disappearance *of administered amines were studied during* degeneration in animals pretreated with a large dose of reserpine Of special significance is the finding that terminals showing about the same frequency and distribution at the *various* degeneration times as the type N terminals in untreated animals showed an efficient uptake and a very high capacity to concentrate the amines

No terminals of the D type were seen in the three groups of drug treated animals After nialamide treatment and even more pronouncedly after the administration of NA (nialamide pretreatment) or α methyl NA there appeared however, terminals showing about the same distribution and frequency as the D terminals but *which* had

pronounced varicosities exhibiting a strong fluorescence These terminals — in contrast to the N terminals — showed no or only weak fluorescence in the thin segments of fibre between the varicosities There is thus little doubt that they are identical with the D terminals Terminals with normal axon membrane uptake-concentration mechanism take up and accumulate amines to very high concentrations, both in the varicosities, where the amine granules are highly concentrated (*cf* Norberg and Hamberger 1964), and in the segments between The fact that the D terminals showed an amine accumulation only in the varicosities is strong evidence that this mechanism had been lost but that the granules could still take up amines. The results obtained in the experiments with amine uptake in the reserpinized animals strongly support this view No amine accumulation occurred in terminals of the D type but was observed in those of N type Since reserpine blocks the storage function of the granules but not the axon membrane mechanism, this furnishes independent evidence that the degenerating terminals first lose the latter mechanism and then shortly afterwards the granule uptake mechanism

The administered amines accumulating in the intact adrenergic nerves of normal or reserpinized animals remain for many hours, provided that MAO activity is efficiently inhibited (Malmfors 1965 a) Both accumulated and endogenous amines disappeared quite suddenly after axotomy, however, in spite of a highly efficient MAO inhibition This shows that the disappearance is not due to any intraaxonal degradation by the enzyme The results indicate instead that the transmitter is released from the terminals, as proposed by Benmiloud and Euler (1963) on the basis of quite different observations This view is strongly supported also by the finding that there occurs a distinct mydriasis during the disappearance of NA from the adrenergic nerves in rabbit iris after axotomy (Andén *et al* 1965) This release could be thought to be a consequence of the loss of the uptake-storage mechanisms There is good evidence however that the membrane surrounding the axons of intact adrenergic nerves effectively prevents the transmitter from leaking out even if the reabsorption mechanism of this membrane is strongly inhibited (Malmfors 1965 a) The rapid transmitter release is thus in all probability due mainly to a quite sudden change in the axon membrane which makes possible a passive leakage This change must occur at about the same time as when the axon membrane reabsorption mechanism deteriorates and it would seem to be responsible for the initial disappearance when most of the transmitter is rapidly lost An interesting possibility is that the degenerative processes in the axon membrane give permeability changes leading to *e g* a quite different ionic environment for the amine granules which thereby rapidly lose their capacity to retain their stored amines There is good evidence that amine storage granules are highly sensitive in this respect (Euler 1958 Hillarp 1958 Euler and Lishajko 1961 1963 and Carlsson *et al* 1963

Benmiloud and Euler (1963 studied the effects of bretylium on transmitter depletion following axotomy and made the interesting observation that this drug may reduce or even prevent such depletion It was suggested that bretylium acts by blocking the normal release mechanism This prompted a direct study on the effects of this drug on the adrenergic terminals and their degeneration The finding made by Benmiloud and Euler was fully confirmed Unexpectedly however it was found that the degenerative processes in the terminals which lead to loss of the uptake mechanisms and the stored transmitter occurred just as in untreated animals, with the only clear difference being that their time course as a whole was postponed *i e* the onset of the rapid phases

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On the Mechanism for the Stimulating Action of Reserpine on Acid Secretion in Gastric Fistula Cats

By

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Abstract

Emås, S. *On the mechanism for the stimulating action of reserpine on acid secretion in gastric fistula cats.* Acta physiol. scand. 1965. 64. 224—237. — A single intravenous infusion of reserpine insufficient to reduce antral gastrin activity significantly elicited acid secretion in nonanesthetized cats with innervated and sympathetically denervated stomachs. Atropine or vagotomy reduced the acid response by 55 to 84 per cent. In vagotomized cats atropine produced a smaller but significant reduction. It was concluded that the vagal and sympathetic nerves are not necessary for reserpine to elicit acid secretion but that intact vagi and — to a lesser extent — the postganglionic cholinergic neurons of the vagotomized cats enhance the effect of reserpine. Reserpine treatment has previously been shown to sensitize the parietal cells to gastrin and to cause a transient reduction in antral gastrin activity in nonvagotomized but not in vagotomized cats. In the present study reserpine treatment reduced the secretory response to intravenous reserpine in nonvagotomized cats and abolished the response in vagotomized cats. A single intravenous infusion of reserpine elicited acid secretion in anesthetized cats after vagotomy and resection of the regions known or suspected to contain gastrin. The results suggest a secretagogue action of reserpine which is not mediated by the vagi and by gastrin.

The first papers reporting that a single injection of reserpine stimulated gastric acid secretion (Barrett, Rutledge and Rogie 1954, Plummer *et al.* 1954) have since been widely confirmed, but the mechanism for the stimulating action has not yet been clarified.

According to preliminary communications reserpine elicits acid secretion in the sympathetically denervated stomach (Emås 1961, Schapiro and Woodward 1961) and experimental data in support in this are offered in the present paper. It has also been shown that reserpine produces acid secretion after vagal denervation (Kirsner and Ford 1957, Rider, Moeller and Gibbs 1957, Schneider and Clark 1957, Schapiro and Woodward 1961, Castiau and Reuse 1962, Kim and Shore 1963), but further studies are needed to evaluate the significance of the vagal nerves for the secretory effect of reserpine.

The injection of 0.10 mg of reserpine per kg caused a release of gastrin from the antral mucosa of cats within 12 hrs. The gastrin released was calculated to be sufficient

to maintain the gastric secretion of acid for more than 12 hrs The same dose did not, on the other hand, affect antral gastrin activity after vagotomy A small dose of reserpine (0 030 mg per kg) produced no change of activity in nonvagotomized cats (Emås and Fyrö 1965). If a single infusion of 0 030 mg of reserpine per kg or less elicits acid secretion in vagotomized cats, this would accordingly indicate a stimulatory effect of reserpine independently of the vagi and the antral gastrin A reduced secretory response to reserpine was found to persist after vagotomy in the present study Since it has been suggested that reserpine evokes secretion by stimulating the parasympathetic ganglia (Barrett *et al* 1955, Kim and Shore 1963), the effect of atropine on reserpine induced secretion was investigated in vagotomized cats

The treatment with reserpine of nonanesthetized cats (0 10 mg per kg i m. daily) has previously been shown to sensitize the HCl secreting cells to infused gastrin (Emås 1963, 1964 a) and to reduce antral and duodenal gastrin activity (Emås and Fyrö 1965) Vagotomy abolished these effects (Emås 1964 b, Emås and Fyrö 1965) By using nonvagotomized and vagotomized cats, it was then possible to study the influence of reserpine treatment, with and without its sensitizing and gastrin-releasing effects, on the acid response to a single infusion of reserpine Further evidence was obtained for a stimulatory effect of reserpine on acid secretion independently of the vagal nerves and gastrin

Methods

Operative Procedures

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The completeness of the vagotomy was tested by regular insulin in at least 2 expts on each animal, as previously described (Emås 1964 b) After the reoperation of 2 animals vagotomy was considered complete in all 9 cats The last test with insulin was carried out 5 to 12 months after vagotomy in 4 animals, with no signs of vagal reinnervation. In the remaining cats the interval was shorter

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1960) The vagal and sympathetic trunks were cut in the neck and the distal part of the stomach was resected from a level oral to the pyloric sphincter by 6 cm on the lesser and 7 cm on the greater curvature The anatomical antrum-corpus border was situated in the resected segment Since gastrin-like activity has been demonstrated in the proximal duodenum of cats (Emås and Fyrö 1965) and has been claimed to be present in the atrophic pancreas of dogs (Elliott *et al* 1963) 16 cm of the proximal duodenum and the pancreas was resected after ligation of the duodenum and the common bile duct The presence of a small gastrin like activity in the cardiac mucosa of hogs (Gregory and Tracy 1961) was not confirmed in cats (Emås and Fyrö unpublished

Experimental Technique

At least 2 weeks were allowed after operation before the secretory studies were started on the nonanesthetized cats Experiments on the anesthetized cats were started 1—2 hrs after completion of the operation and the rectal temperature was maintained between 37 and 38° C throughout each experiment by means of an infrared lamp The animals were deprived of food and liquid for 18 to 24 hrs before each experiment.

The volume of gastric juice was recorded for 15 min periods and the amount of free and total acid determined by titration against 0 01 N NaOH, with Topfer's reagent and phenolphthalein as indicators The amount of acid secreted was expressed in meq

the study that the infusion of reserpine tended to reduce the secretory response to a subsequent infusion 3 to 5 days later Experiments on the same animal were therefore performed at intervals of at least one week except in reserpinized cats (see below), in which shorter intervals were allowed

In the nonvagotomized cats, the amounts of reserpine infused i v ranged from 0 010 to 0 030 mg per kg b w for 15 min and were individually adjusted to produce a submaximal secretory response of acid Unless otherwise stated, the total dose for each cat was kept constant throughout the series, despite fluctuations in b w In the anesthetized cats, 0 060 or 0 080 mg per kg was infused for 15 min The secretory rate was in all experiments far from the maximal secretory capacity of the cat's stomach (*cf* Emås 1960, 1963) The doses of atropine (as sulphate) ranged from 0 05 to 0 5 mg per kg of b w

response to a single infusion of reserpine was accordingly defined as meq of total acid secreted *during 5 hrs from the start of the infusion, minus 5 times the 1-hr basal secretion*

The reduction in acid output following atropine was calculated as the difference between the mean 15 min output of total acid (uncorrected) during the 30-min period before the injection of atropine (control level) and that during the 30 min period starting 1 hr after injection In the statistical analysis (see below), the differences were compared with corresponding differences in the experiments with reserpine alone on the same animal

In 2 ordinary gastric fistula cats, secretion was recorded for 12 hrs following the infusion of reserpine In about half the number of experiments on each animal, the fluid loss caused by the hypersecretion was partially compensated for by the continuous i v infusion of saline at a rate of 0 11 ml/min (infusion pump) The saline infusions started in most experiments 6 hrs after reserpine and continued for at least 4 hrs

Reserpine Treatment

Reserpine was injected i m in a dose of 0 10 mg per kg b w every 24 hrs for periods of usually 3 to 11 days (mean 8 days) After reserpine treatment for 3 days or more the animals are referred to as *reserpinized* (Emås 1963) *The secretory studies were carried out* every 2nd or 3rd day on the reserpinized animal each experiment starting 20 to 24 hrs after the previous reserpine injection On days when experiments with reserpine were performed the regular i m dose of reserpine was reduced to obtain a total daily dose of 0 10 mg per kg Each animal was subjected to 1 to 4 periods of reserpinization Further details have been given elsewhere (Emås 1963)

Experiments performed 24 hrs after a single i m injection of 0 10 mg of reserpine per kg (reserpine pretreatment) are accounted for separately

Evaluation of Data

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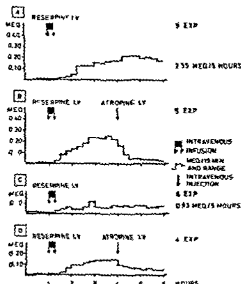


Fig 1 Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine in a nonanesthetized gastric fistula cat (no 86)
A Before vagotomy Dose of reserpine 0 025 mg per kg of b.w for 15 min Corrected mean secretory response 2 55 meq per 5 hours
B Before vagotomy Effect of i v atropine (0 50 mg per kg of b w) on the secretory response Dose of reserpine as in A
C After vagotomy Dose of reserpine as in A Corrected mean secretory response 0 93 meq per 5 hours
D After vagotomy Effect of i v atropine (0 05 mg per kg of b w) on the secretory response Dose of reserpine 0 050 mg per kg of b w for 15 min

Results

A Experiments on Ordinary Gastric Fistula Cats

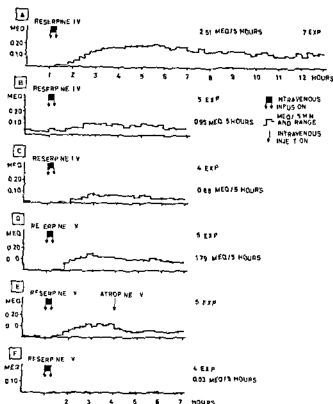
Secretory Response to a Single Reserpine Infusion

11 cats were used and 4 or 5 expts were performed on each animal After 30 to 60 min from the start of the reserpine infusion (0 010 to 0 030 mg per kg b w) acid secretion gradually increased reaching about maximum rate during the 3rd post infusion hour and remaining then almost constant for 2 to 3 hrs before slowly subsiding The mean responses of 2 representative cats are shown in Fig 1 A and 2 A The mean peak output of total acid varied among the 11 cats from about 0 10 to 0 40 meq per 15 min, these variations being due to the different reserpine doses employed and to the different susceptibility of the cats to reserpine During the period of maximum secretion the curves for acid output in the experiments on the same animal had an almost horizontal course although at different levels Due to the small variation in acid output during maximum secretion within experiments the ranges in the figures reflect mainly the differences in secretory levels as between experiments

In 2 cats investigated mean acid output during the 12th hour after the infusion of 0 020 (Fig 2 A) or 0 010 mg (4 expts) of reserpine per kg amounted to about 50 per cent of maximum or 0 10 meq of total acid per 15 min The continuous i v infusion of saline for 4 hrs or more did not influence the slow decline in acid secretion

Effect of Atropine on the Secretory Response to a Single Reserpine Infusion

Following i v atropine inhibition appeared within 15 min reached its maximum in about 60 min, and remained throughout the experiments 2 or 3 hrs The inhibition of acid secretion was never complete In 3 cats (no 81, 82, and 86) 0 50 mg of atropine per kg reduced the mean 15-min output of total acid by 0 18 2 expts 0 17 (4 expts),



...sion of
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Corrected mean secretory response 0.9.. meq per 5 hours

C After vagotomy Dose of reserpine as in A Corrected mean secretory response 0.88 meq per 5 hours

...

F During reserpine treatment Preparation of the animal and dose of reserpine as in D Corrected mean secretory response 0.03 meq per 5 hours

and 0.20 meq (Fig 1 B) or 75 to 83 per cent (mean control levels 0.24 0.22, and 0.24 meq) In 2 other cats (no 84 and 89) 0.25 mg of atropine per kg reduced the mean acid output by 0.20 meq or 65 per cent (4 expts mean control level 0.31 meq) and by 0.14 meq or 70 per cent (3 expts, mean control level 0.20 meq) The inhibition following 0.50 and 0.25 mg per kg was highly significant ($P < 0.001$) After 0.10 mg of atropine per kg (cat 89) the mean reduction was 0.12 meq of total acid ($P < 0.05$, almost significant) or 55 per cent (2 expts, mean control level 0.22 meq) In 3 to 5 expts with reserpine alone on each animal there was no apparent decline in mean acid output during the 4th and 5th hours after reserpine

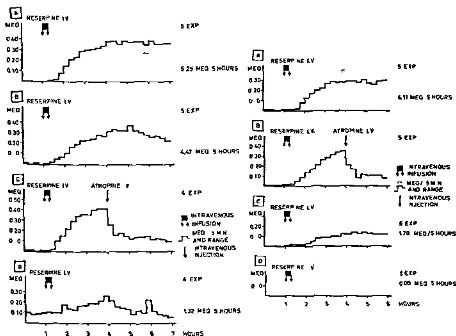


Fig 3 (left) Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine (0 030 mg per kg of b w for 15 min) in a nonanesthetized gastric fistula cat (no 89)

A After preganglionic sympathectomy Corrected mean secretory response (during the period from 1 to 6 hours) 5 25 meq per 5 hours

B After pre- and postganglionic sympathectomy Corrected mean secretory response 4 47 meq per 5 hours

C After pre and postganglionic sympathectomy Effect of i v atropine (0 10 mg per kg of b w) on the secretory response

D During reserpine treatment Preparation of the animal as in B Corrected mean secretory response 1 32 meq per 5 hours

Fig 4 (right) Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine (0 030 mg per kg of b w for 15 min) in a nonanesthetized gastric fistula cat (no 71)

A After pre and postganglionic sympathectomy Corrected mean secretory response 4 11 meq per 5 hours

B After pre and postganglionic sympathectomy Effect of i v atropine (0 10 mg per kg of b w) on the secretory response

C After pre and postganglionic sympathectomy and vagotomy Corrected mean secretory response 1 70 meq per 5 hours

D During reserpine treatment Preparation of the animal as in C Corrected mean secretory response 0 00 meq per 5 hours

Secretory Response to a Single Reserpine Infusion during Reserpine Treatment

With the 3 cats used (no 98 155 and 162) reserpinization increased the mean 1 hr basal output of total acid by 0 03 to 0 11 meq

In cat 98 reserpinization reduced the mean 5 hr response to a single reserpine infusion by 1 56 meq of total acid (Fig 2 A and B) or 62 per cent The mean response

to 0 030 mg of reserpine per kg amounted in cat 155 to 1 87 meq per 5 hrs (4 expts) in the nonreserpinized state and 1.51 meq (4 expts) in the reserpinized state, and in cat 162 to 5 47 (5 expts) and 2 40 meq (5 expts), respectively Reserpinization thus reduced the mean response of cats 155 and 162 by 19 and 56 per cent The responses of the nonreserpinized and reserpinized cats differed highly significantly ($P < 0\,001$) The size of the reduction was not related to the length (3 days or more) of the reserpinization period

The figures for percentage reduction were approximately the same when calculated on acid output during the 5th post-infusion hour instead of on the 5 hr secretory response

B Experiments on Sympathectomized Gastric Fistula Cats

The experiments on cat 39 started 4 months after preganglionic sympathectomy and were carried out within a period of 2 months, and on cat 71 5 months after pre- and postganglionic sympathectomy and were completed in 6 months On cat 89, experiments were performed both after preganglionic and after pre- and postganglionic sympathectomy The former experiments started 15 days, the latter 8 months after the operation and were completed in 5 and 8 months respectively

Secretory Response to a Single Reserpine Infusion

The curves for acid output following the infusion of reserpine (0 025 or 0 030 mg per kg) had the same shape in the preganglionically (no 39 and 89) and the pre- and postganglionically sympathectomized cats (no 71 and 89) as in ordinary gastric fistula cats, and the 5-hour responses fell within the ranges of the latter The mean output of total acid reached in cat 39 a maximum level of 0 25 meq per 15 min (4 expts) during the 3rd hour after reserpine (0 025 mg per kg), remaining then almost constant for the last 2 hrs The mean responses of cat 89 and 71 are shown in Fig 3 A and B and Fig 4 A, respectively In cat 89 the responses after preganglionic (Fig 3 A) and after pre- and postganglionic sympathectomy (Fig 3 B) did not differ significantly ($P > 0\,05$)

Effect of Atropine on the Secretory Response to a Single Reserpine Infusion

In a preganglionically sympathectomized cat (no 39) 0 50 mg of atropine per kg reduced the mean 15-min output of total acid by 0 11 meq ($P < 0\,05$) or 58 per cent (4 expts, mean control level 0 19 meq), while in 2 pre- and postganglionically sympathectomized cats (no 71 and 89) 0 10 mg of atropine per kg reduced the mean acid output by 0 24 meq or 69 per cent (Fig 4 B) and by 0 28 meq or 67 per cent (Fig 3 C), a highly significant ($P < 0\,001$) reduction Complete inhibition was never observed The experiments with reserpine alone are reported above

Secretory Response to a Single Reserpine Infusion during Reserpine Treatment

Experiments were performed on cat 89 after preganglionic and after pre- and postganglionic sympathectomy and gave similar results as those on ordinary gastric fistula cats

After preganglionic sympathectomy, reserpinization (2 expts) increased the mean 1-hr basal output of total acid by 0 16 meq but reduced the mean 5-hr response to i v reserpine by 4 48 meq ($P < 0\,001$) or 85 per cent The mean response of the nonreserpinized animal is illustrated in Fig 3 A

TABLE I Mean 5 hour secretory response to intravenous infusions of reserpine in nonanesthetized gastric fistula cats before and after bilateral vagotomy

		Before vagotomy		After vagotomy		Decrease	
Cat no	Reserpine in mg/kg	No of expts	Response[1] in meq	No of expts	Response[1] in meq	in meq	in per cent
29	0.010	4	1.92 [2]0.77—2.96	5	0.73 [2]0.49—0.92	1.19	62
41	0.010	4	1.39 0.58—2.20	4	0.48 0.17—1.25	0.91	65
[3]86	0.025	5	2.55 1.98—3.18	4	0.93 0.36—1.36	1.62	64
[4]98	0.020	7	2.51 1.89—2.90	4	0.88 0.45—1.39	1.63	65
155	0.030	4	1.87 1.60—2.21	1	0.30	1.57	84
Pre- and postganglionically sympathectomized							
[5]71	0.030	5	4.11 2.03—5.69	5	1.70 1.19—2.43	2.41	59

[1] Correction made for basal secretion
[2] Range of secretory responses
[3] Illustrated in Fig 1 A and C
[4] Illustrated in Fig 2 A and C
[5] Illustrated in Fig 4 A and C

After pre and postganglionic sympathectomy the increase in the mean 1 hr basal output of total acid during reserpinization amounted to 0.35 meq and the reduction of the mean response to i v reserpine to 3.15 meq or 70 per cent (Fig 3 B and D) This reduction was significant ($P < 0.01$)

C Experiments on Vagotomized Gastric Fistula Cats

In 8 of 9 cats the experiments with reserpine started within less than 3 months and in the remaining cat 6 months after complete vagotomy The experiments were completed in 4 months except for cat 94 in which the last experiment was performed 16 months after vagotomy Cat 71 had been pre and postganglionically sympathectomized before the vagotomy

Secretory Response to a Single Reserpine Infusion

Reserpine (0.010 to 0.060 mg per kg) produced usually within 1 hr a gradual increase of gastric acid secretion (Figs 1 C, 2 C, and 4 C) in all 9 vagotomized cats The maximum rate of acid secretion was reached during the 3rd or 4th post infusion hour and then remained approximately constant or declined slightly during the subsequent 2 to 3

hrs The mean peak acid output varied among the animals from about 0 05 to 0 15 meq per 15 min

In the 6 cats investigated, vagotomy reduced the mean 5-hr response (Table I) by 0 91 to 2 41 meq, or 59 to 84 per cent This reduction was highly significant ($P < 0\ 001$) The experimental data of cat 71 were not included in the statistical analysis Fig 1, 2, and 4 (A and C) show the mean responses of 3 cats before and after vagotomy

Effect of Atropine on the Secretory Response to a Single Reserpine Infusion

In 3 vagotomized cats 0 25 mg of atropine per kg reduced the reserpine-induced acid secretion highly significantly ($P < 0\ 001$), in cat 29 the mean 15 min output of total acid (0 015 mg of reserpine per kg) was reduced by 0 03 meq or 27 per cent (3 expts, mean control level 0 11 meq), in cat 98 by 0 06 meq or 52 per cent (Fig 2 E), and in cat 165 (0 060 mg of reserpine per kg) by 0 07 meq or 62 per cent (5 expts, mean control level 0 11 meq) In one cat investigated, 0 05 mg of atropine per kg reduced acid output by 0 09 meq ($P < 0\ 01$) or 61 per cent (Fig 1 D) 4 or 5 expts were performed with reserpine alone on each animal

Secretory Response to a Single Reserpine Infusion during Reserpine Treatment

The 5-hr secretory response to i v reserpine was determined in 4 cats, nonreserpinized and reserpinized Reserpinization had no influence on the basal output of total acid, but eliminated the secretory response to i v reserpine in all cats The mean 5 hr responses of cat 71, nonreserpinized and reserpinized are shown in Fig 4 C and D, and of cat 98 in Fig 2 D and F The mean response in cat 94 (0 030 mg of reserpine per kg) amounted in the nonreserpinized state to 3 93 meq (6 expts) and in the reserpinized state to 0 06 meq (5 expts), and in cat 165 (0 060 mg per kg) to 1 77 meq (5 expts) and less than 0 01 meq (4 expts) respectively

The variation in responses was much greater in the nonreserpinized than in the reserpinized animal making the ordinary analysis of variance inappropriate There was no doubt, however, as to the effect of reserpinization since the reduction was very uniform and pronounced

Reserpine treatment for 3 days or more reduced or even eliminated (in the vagotomized cats) the secretory response to i v reserpine In a series of experiments the 5 hr secretory responses to i v reserpine were determined also 24 hrs after a single i m injection of 0 10 mg of reserpine per kg (pretreatment) In one ordinary gastric fistula cat (no 98) reserpine pretreatment reduced the mean response (2 expts) by 0 72 meq or 29 per cent while in the other (no 162) no reduction occurred (3 expts) Reserpinization for 3 days reduced the mean response in these animals by about 60 per cent (see Section A) In cat 89 (pre and postganglionically sympathectomized) a single reserpine injection reduced the mean response (6 expts) to i v reserpine by 1 65 meq (P [illegible] not significant) or 37 per cent while reserpinization for 3 days reduced the mean response by 70 per cent (see Section B) In 11 expts on 3 vagotomized cats reserpine pretreatment reduced the response to i v reserpine by 1 31 (cat 98) to 2 09 meq (cat 94) or 53 (cat 94) to 76 per cent (cat 165) — a highly significant (P [illegible] 0 001) reduction — but in contrast to reserpinization (see Section C) it never actually abolished the response

In the nonvagotomized cats pretreatment with reserpine increased basal acid secretion to about the same extent as reserpine treatment for 3 days or more Reserpine pretreatment did not alter basal secretion in the vagotomized cats

D Experiments on Anesthetized Gastric Fistula Cats Subjected to Vagotomy and Resection of Gastrin Releasing Regions

Secretory Response to a Single Reserpine Infusion

The 1 hr basal secretion contained no titratable free acid and total acid amounted to 0 01 meq or less

A single i v reserpine infusion (0 060 or 0 080 mg per kg) elicited acid secretion within 1 hr in all 7 cats The curves for total acid output had in principle the same shape as those obtained from the nonanesthetized cats with vagally denervated stomachs The peak output of total acid was reached in the 3rd or 4th post infusion hour and varied among the cats from approximately 0 05 to 0 30 meq per 15 min The 5-hr response ranged from 0 43 to 4 81 meq of total acid (mean 2 20 meq)

Discussion

Intravenous reserpine has been shown to stimulate gastric acid secretion in humans (Beman Knoll and DeLor 1955 Clark and Schneider 1955 Haverback *et al* 1955 Krogsgaard 1955) nonanesthetized dogs (Barrett Rutledge and Rogie 1954) and anesthetized cats (Gaitonde Satoskar and Mandrekar 1960) In the present study on nonanesthetized cats a single i v reserpine infusion elicited a gastric secretion of acid which appeared after a delay of 30 to 60 min and lasted for at least 12 hrs

In agreement with previous reports (Emås 1961 Schapiro and Woodward 1961) the sympathetic nerves to the stomach appear to be inessential to the stimulating effect of reserpine on acid secretion since the secretory response to reserpine was about as great in sympathectomized as in ordinary gastric fistula cats The difficulty of performing a complete and permanent sympathetic denervation of the stomach has been emphasized previously (Emås 1964 a)

Injected reserpine stimulates acid secretion in the vagally denervated stomach of humans (Kirsner and Ford 1957 Rider Moeller and Gibbs 1957 Schneider and Clark 1957) dogs (Schapiro and Woodward 1961 Castiau and Reuse 1962) and rats (Kim and Shore 1963) Only a few data have been reported illustrating the effect of vagotomy on the secretory response to reserpine Schneider and Clark (1957) reported that vagotomy did not significantly alter the acidity of the response in 2 human subjects Kim and Shore (1963) obtained a profound reduction of the acid response in non anesthetized rats which they ascribed to the vagotomy greatly inhibiting the reserpine-induced release of gastric histamine It has been suggested that a parasympathetic mechanism is implicated in the stimulating action of reserpine on acid secretion since anticholinergic drugs reduced the acid response in nonanesthetized dogs with vagally innervated stomachs (Barrett Rutledge and Rogie 1954 La Barre and Lieber 1957) Corresponding studies on humans however have given varying results (for references see Levrat and Lambert 1962) and in anesthetized dogs even large doses of atropine (2 mg per kg i v) failed to reduce the reserpine-induced secretion (Castiau and Reuse 1962) In the present investigation vagotomy reduced the response to reserpine in nonanesthetized cats by 59 to 84 per cent and about as large a reduction was produced by atropine (0 10 to 0 50 mg per kg) in nonvagotomized cats The reduction seems mainly to reflect a reduced excitability of the HCl-secreting cells due to the elimination or inhibition of vagal sensitizing impulses to the parietal cells (Emås 1964 b) since in 3 out of 4 cats used also in a previous study (Emås 1964 b) vagotomy

produced approximately the same percentage reduction of the responses to infused histamine and infused gastrin extracts A blockade or reduction of a vagal and gastrin releasing effect of reserpine (see next paragraph) might, however, also have contributed to the reduction Neither vagotomy nor atropine abolished the secretory response to reserpine completely The stimulating effect of a single reserpine infusion on acid secretion therefore differs from the effect of reserpine treatment on acid secretion (Emås 1964 b) and from that of reserpine on antral and duodenal gastrin activity (*Emås and Fyro 1965*), *in that it does not require intact vagal nerves In nonanesthetized cats*, however, intact vagal nerves are essential for a single reserpine infusion to exert an optimal stimulating effect

A previous study (Emås and Fyro 1965) demonstrated that 0 10 mg of reserpine per kg i m produced a reduction of antral gastrin activity in nonvagotomized cats while no measurable reduction occurred in nonvagotomized cats after 0 030 mg per kg or in vagotomized cats after 0 10 mg per kg The secretory response of the non vagotomized cats to the relatively small amounts of reserpine infused in the present study (0 010 to 0 030 mg per kg) could therefore be due, at least in part, to an action of reserpine that does not involve gastrin release In the vagotomized cats the vagal preganglionic neurons can be excluded, and gastrin seems an unlikely mediator for the stimulating action of a single reserpine infusion on acid secretion An action of reserpine on acid secretion independently of the vagal nerves and the gastrin mechanism was supported also by the finding that a single reserpine infusion elicited a significant response of acid in anesthetized, vagotomized cats, in which the gastrointestinal regions known or suspected to contain gastrin had been resected Moreover, there was no close parallelism in nonanesthetized cats between the effects of reserpine treatment or pre treatment on the secretory response to i v reserpine and on the antral gastrin activity In nonvagotomized cats, reserpine treatment significantly reduced both the secretory response to i v reserpine — this has been observed also in two human subjects (Bachrach 1959 Haverback and Wirtschafter 1962) — and the antral gastrin activity (Emås and Fyro 1965), but *increased the susceptibility of the HCl secreting cells to exogenous* histamine and gastrin (Emås 1963 1964 a) In vagotomized cats reserpine treatment abolished the response to i v reserpine without altering either the antral gastrin activity (Emås and Fyrö 1965) or the susceptibility of the HCl-secreting cells to hist amine and gastrin (Emås 1964 b) Pretreatment with reserpine 24 hours before the experiment on the other hand, did not significantly alter the acid response to i v reserpine in nonvagotomized cats although it reduced antral gastrin activity as much as 2 or 4 days of treatment (Emås and Fyro 1965) It decreased the response significantly however in vagotomized cats

The latent period and long duration of the secretory response to reserpine in addition to the reduction of the response by reserpine treatment, suggest that the non vagal and non-gastrin component of reserpine action elicits acid secretion by an intermediate mechanism. The acid response is reported to persist after adrenalectomy in dogs (Schapiro and Woodward 1961 Castiau and Reuse 1962) but no experimental data were presented The persistence of the response demonstrates that the adrenals are not essential for the stimulatory action of reserpine on acid secretion It offers however, no information concerning a cooperation of the adrenals since the vagal innervation [illegible] (Emås and Fyrö 1965)

[illegible] Vaalker

[illegible] sterholm

1963) The finding of Kim and Shore (1963) could indicate that reserpine caused a release of gastric histamine in rats mainly by a vagal action Whether histamine is the mediator for the non vagal and non gastrin component of reserpine action cannot be excluded however

Barrett *et al* (1955) suggested that reserpine evokes gastric acid secretion by stimulating the parasympathetic ganglia since an anticholinergic and a ganglionic blocking drug inhibited the reserpine induced secretion in the vagally denervated (Heidenhain) gastric pouch of dogs In this pouch preparation the vagal supply of the antral and duodenal mucosa is left intact, which makes gastrin release by reserpine possible (Emås and Fyro 1965) Kim and Shore (1963) reported that the ganglionic blocking drug chlorisondamine was more effective than vagotomy in reducing the acid response of nonanesthetized rats to reserpine The small but significant reduction of reserpine-induced acid secretion by atropine in the vagotomized cats might appear to support the suggestion of Kim and Shore that part of the secretory response to reserpine is produced by ganglionic stimulation However, this hypothesis for reserpine action seems difficult to reconcile with the finding that the reserpine treatment of vagotomized cats abolished the secretory response to a single reserpine infusion but did not alter the *excitability of the HCl secreting cells* (Emås 1964 b) Furthermore, in vagotomized cats the acid secretion elicited by a single reserpine infusion and by a continuous i v infusion of histamine or of gastrin preparations was susceptible to inhibition by atropine, and reserpine and histamine induced secretion were about equally reduced by 1 mg of chlorisondamine (Ecolid®) per kg b w i v (Emås unpublished observations) The two inhibitors were tested at approximately the same submaximal secretory rate Since cholinergic excitation increases the responsiveness of the HCl secreting cells to other stimuli (for references see Emås 1964 b) the reductions by atropine and chlorisondamine in the vagotomized cats can possibly be ascribed to the blockade of a sensitizing effect of the postganglionic cholinergic neurons on the HCl secreting cells rather than to a blockade of the effect of ganglionic stimulation

To conclude the present author agrees with the view that a single reserpine infusion elicits acid secretion by intermediate mechanisms and not by an action directly on the HCl secreting cells Evidence of a vagal action of a relatively large dose of reserpine (0 10 mg per kg) causing gastrin release has previously been published (Emås and Fyro 1965) The present investigation demonstrates that an intact sympathetic and vagal supply of the stomach is not necessary for reserpine (0 01 to 0 03 mg per kg) to stimulate acid secretion but that intact vagal nerves are necessary for reserpine to exert its optimal stimulatory effect The reduction of the response by atropine in the nonvagotomized cats and by vagotomy is assumed to be due mainly to the inhibition of vagal sensitizing impulses to the HCl secreting cells (Emås 1964 b) and the small reduction by atropine in the vagotomized cats to the inhibition of a sensitizing effect of acetylcholine released from the postganglionic cholinergic neurons In favour of a secretagogue action of reserpine which is not mediated by the vagal nerves and the gastrin mechanism are the findings (1) that the i v infusion of relatively small amounts of reserpine elicited acid secretion in both nonvagotomized and vagotomized cats but produced no measurable reduction of antral gastrin activity in nonvagotomized cats and 3 to 10 times these amounts produced no measurable reduction of activity in vagotomized cats (Emås and Fyrö 1965) (2) that 3 days or more of reserpine treatment reduced and abolished the secretory response to i v reserpine in nonvagotomized and vagotomized cats respectively but reduced antral gastrin activity only in the nonvagotomized cats Emås and Fyro

1965), (3) that reserpine pretreatment of nonvagotomized cats did not significantly alter the secretory response to i v reserpine, but reduced antral gastrin activity as much as 2 or 4 days of reserpine treatment (Emås and Fyro 1965) and (4) that i v reserpine elicited acid secretion in anesthetized cats subjected to vagotomy and resection of regions known or suspected to contain gastrin

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Renal Clearances of Inulin, Polyfructosan-S and a Polyethylene Glycol (PEG 1,000) in the Rat

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Abstract

Berglund F *Renal clearance of inulin polyfructosan-S and a polyethylene glycol (PEG 1,000) in the rat* Acta physiol scand 1965 64 238—244 The renal clearances of inulin, polyfructosan-S and PEG 1 000 were measured in the rat by means of a constant infusion technique The inulin/PEG 1 000 clearance ratio in 10 rats averaged 0.81, and polyfructosan-S/PEG 1 000 ratio in 10 rats averaged 0 87 The clearance ratios differed signicantly from unity and were not influenced by phlorizin The restricted filterability of the two polysaccharides in the rat is attributed to the pore size of the glomerular membrane, and a relationship between pore size and animal size is suggested

The clearance of inulin has generally been considered to be the most reliable measure of glomerular filtration rate (GFR) since its introduction for this purpose in 1934 In certain species the clearance of other unrelated substances may equal that of inulin, and thus presumably equal GFR In the dog this applies to creatinine (Shannon 1936), ferrocyanide ion (Berliner, Kennedy and Hilton 1950), glucosamine (Carter and Peters 1958), polyethylene glycols of molecular weight up to 4 000 (Shaffer, Critchfield and Carpenter 1948), and dextran of molecular weight up to 4,000 (Wallenius 1954)

In a recent attempt to measure renal tubular reabsorption of inorganic sulfate in the rat, a reabsorption + secretion pattern was obtained, evidently because inulin clearances were lower than GFR When GFR was measured with low molecular weight polyethylene glycol (PEG 1 000 or PEG 400) instead of inulin a regular Tm pattern was obtained for sulfate reabsorption (Berglund 1964)

In the present paper the clearances of two fructans, inulin and polyfructosan-S, are compared with the clearance of PEG 1 000 in the rat

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Methods

Clearance experiments were done on unanaesthetized male blackhooded rats by a constant infusion technique (Berglund 1964) Each experiment consisted of six clearance periods Test substances were inulin derived from chicory (L Light & Co, Ltd) polyfructosan-S (Laevosan Gesellschaft) and PEG 1 000 (Union Carbide)

Inulin and polyfructosan-S were analysed by the method of Heyrovsky (1956), and PEG 1,000 by the method of Hydén (1955) as modified by Berglund (1964)

Results

Clearances and clearance ratios are listed in Table I The inulin clearance (0 78 ± 0 14 ml/min/100 g) was significantly higher than reported by earlier authors (cf Smith 1951, Fingl 1952), with the exception of Peters (1959) (0 70 ± 0 21 ml/min/100 g)

Inulin/PEG 1 000 clearance ratios averaged 0 81, and polyfructosan-S/PEG 1,000 clearance ratios averaged 0 87 The difference between these ratios is not statistically significant but both ratios differed significantly from unity The inulin/PEG clearance ratio was well below 1 0 even when arterial blood samples were used for analysis (exp 334)

In one rat, inulin/PEG 1 000 clearance ratios were measured in two consecutive experiments using non boiled inulin (exp 333), and inulin which had been kept in boiling water for 1 hour (exp 332) The boiled inulin showed a higher clearance ratio than the non boiled

Phlorizin (exp 307) did not raise a low polyfructosan S/PEG 1 000 clearance ratio significantly This should rule out tubular reabsorption of polysaccharide as a possible source of error

Discussion

The low polysaccharide/PEG 1 000 clearance ratios in the rat may be related to various factors, such as molecular weight and molecular inhomogeneity of the test substances as well as the average pore size of the glomerular membranes These points will be discussed especially in relation to inulin

Inulin is a D fructose polymer or fructan containing small amounts of D-glucose, probably in a sucrose type linkage at the reducing end of the molecule It forms the major polysaccharide in tubers of many *Compositae* Most commercial inulin seems to derive from dahlia (*Dahlia rosea*) but at least three brands L Light & Co Ltd Laevosan-Gesellschaft E Merck A G) derive from chicory (*Cichorium Intybus*

For the molecular weight of inulin the figure 5100 (Westfall and Landis 1936 is quoted in textbooks of renal physiology Smith 1951 Pitts 1963 in spite of higher figures in the literature since 1936 Table II Molecular weight does not seem to vary significantly between inulins from different genera Highly divergent molecular weights may however be obtained before and after fractionation *e g* with ethanol The figures given by Lenaka 1951) and by Vink 1960 are especially noteworthy in this respect (*cf* Table II and indicate a considerable degree of molecular inhomogeneity of inulin This has also been demonstrated by paper electrophoresis and by differential precipitation with ethanol Bassir 1956 The continuous fall in inulin clearance after a single intravenous injection in man may also be explained by inhomogeneity high molecular weight fractions supposedly being excreted slower than the low molecular weight fractions Ferguson *et al* 1950 Barnard Bassir and Hough 1955)

TABLE I Clearance ratios (inulin/PEG 1,000 or polyfructosan S/PEG 1,000) in rats

Exp	Weight g	Plasma fructan mg %	Plasma PEG mg %	Fructan clearance ml/min/100 g	PEG clearance ml/min/100 g	Fructan/PEG clearance ratio
"Fructan" = Inulin						
259	341	54	82	0 61	1 03	0 61
260	255	63	103	0 71	0 98	0 72
327	240	33	86	1 04	1 31	0 81
332	257	72	Inulin in 100°C water bath for 60 min Same rat as exp 33			
			134	0 44	0 51	0 87±0 0
333	257	41	97	0 67	0 94	0 72±0 07
334	250	44	75	0 39	0 60	0 65
339	300	36	62	0 88	1 09	0 83
340	335	41	71	0 74	0 73	1 03
341	345	36	69	0 85	0 81	1 05
342	360	24	88	0 65	0 82	0 81
343	325	31	55	0 87	1 06	0 83
"Fructan" Polyfructosan S						
305	245	56	145	0 69	0 81	0 85
307	228	88	67	0 84	1 30	0 64
		Phlorizin 44 mg/kg i v		0 75	1 11	0 67
308	300	72—114	106	0 93	1 14	0 83
310	248	64	149	0 70	1 06	0 66
311	290	44	101	1 13	1 22	0 89
312	250	44	85	1 17	1 22	0 96
313	290	45	100	1 12	1 13	1 00
315	275	54	200	1 01	0 93	1 04
316	275	97	160	0 73	0 82	0 89
338	342	47	56	1 07	1 15	0 93

Notes Exp 307 phlorizin data not included in statistical treatment
Exp 327 dahlia inulin (British Drug Houses Ltd)
Exp 332 included only in statistical treatment 5
Exp 334 anesthesia with Inactin[®] Na 5-ethyl 5-(1 methyl propyl) thiobarbiturate Blood samples from carotid artery Included only in statistical treatment 4

Statistical treatment (means ± standard deviations)

1 Clearance of inulin	0 78 ± 0 14 ml/min 100 g	n	9
2 Clearance of polyfructosan S	0 94 ± 0 19 ml min 100 g	n	10
3 Clearance of PEG 1 000			
inulin series	0 97 ± 0 19 ml min 100 g	n	9
polyfructosan S series	1 03 ± 0 17 ml min 100 g	n	10
combined	1 03 ± 0 18 ml min/100 g	n	19

Table II Molecular weight of inulin Data from 1936 and later

Source of inulin	Method	Mol wt.	Reference
Dahlia recrystallized twice	Thermoelectric vapor pressure	4 457	Westfall and Landis 1936
Recrystallized 5 times		5 101	
Inula helenum [4] repeated purification	Thermoelectric vapor pressure	7 777	Bezzi 1939
"Blue Danube" dahlia tubers	Analysis of hydrolysis products	6 150	Hirst et al 1950
Taraxacum officinale	Glucose-analysis with rotation	6 300	Palmer 1951
Dahlia rapidly crystallizing		6 800	
Dahlia slowly crystallizing		7 290	
Inula helenum		6 800	
Kahlbaum original	Osmometer cellophane membrane	5 200	Uenaka 1957
Repeated ethanol fractionation		7 000	
Cichorium Intybus before ethanol fractionation	Osmometer viscose membrane	3 800	Vink 1960
3rd precipitate		4 900	
12th precipitate		7 700	

4 Clearance ratios

a) inulin/PEG 1 000	0 81 ± 0 14	n 10
b) polyfructosan S/PEG 1 000	0 87 ± 0 13	n 10
Comparison a) vs b)	p > 0 20	
Comparison a) vs. unity	p < 0 005	
Comparison b) vs unity	p < 0 025	

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Exp 332 clearance ratio	0 87 ± 0 05	n 6
Exp 333 clearance ratio	0 72 ± 0 07	n 6
Comparison exp 332 vs. 333	p < 0 001	

TABLE I Clearance ratios (inulin/PEG 1 000 or polyfructosan S/PEG 1,000) in rats

Exp	Weight g	Plasma fructan mg %	Plasma PEG mg %	Fructan clearance ml/min/100 g	PEG clearance ml/min/100 g	Fructan/PEG clearance ratio
"Fructan" = Inulin						
259	341	54	82	0.61	1.03	0.61
260	255	63	103	0.71	0.98	0.72
327	240	33	86	1.04	1.31	0.81
332	257	72	Inulin in 100 C water bath for 60 min Same rat as exp 333			
			134	0.44	0.51	0.87±0.05
333	257	41	97	0.67	0.94	0.72±0.07
334	250	44	75	0.39	0.60	0.65
339	300	36	62	0.88	1.09	0.83
340	335	41	71	0.74	0.73	1.03
341	345	36	69	0.85	0.81	1.05
342	360	24	88	0.65	0.82	0.81
343	325	31	55	0.87	1.06	0.83
"Fructan" = Polyfructosan S						
305	245	56	145	0.69	0.81	0.85
307	228	88	67	0.84	1.30	0.64
		Phlorizin 44 mg/kg i v		0.75	1.11	0.67
308	300	72—114	106	0.93	1.14	0.83
310	248	64	149	0.70	1.00	0.66
311	290	44	101	1.13	1.22	0.89
312	250	44	85	1.17	1.22	0.96
313	290	45	100	1.12	1.13	1.00
315	275	54	200	1.01	0.99	1.04
316	275	97	160	0.73	0.82	0.89
338	342	47	56	1.07	1.15	0.93

Notes Exp 307 phlorizin data not included in statistical treatment
Exp 327 dahlia inulin (British Drug Houses Ltd.)
Exp 332 included only in statistical treatment 5
Exp 334 anesthesia with Inactin® Na 5-ethyl 5-(1 methyl propyl) thiobarbiturate Blood samples from carotid artery Included only in statistical treatment 4

Statistical treatment means ± standard deviations

1 Clearance of inulin 0.78 ± 0.14 ml/min/100 g n = 9
2. Clearance of polyfructosan S 0.94 ± 0.19 ml/min/100 g n = 10
3 Clearance of PEG 1 000
 inulin series 0.97 ± 0.19 ml/min/100 g n = 9
 polyfructosan S series 1.08 ± 0.17 ml/min/100 g n = 10
 combined 1.03 ± 0.18 ml/min/100 g n = 19

TABLE II. Molecular weight of inulin Data from 1936 and later

Source of inulin	Method	Mol wt	Reference
Dahlia, recrystallized twice	Thermoelectric vapor pressure	4,457	*Westfall and Landis* 1936
Recrystallized 5 times		5,101	
Inula helenum "repeated purification"	Thermoelectric vapor pressure	7,777	Berzi 1939
"Blue Danube" dahlia tubers	Analysis of hydrolysis products	6,150	Hirst et al, 1950
Taraxacum officinale	Glucose-analysis with notatin	6 300	Palmer 1951
Dahlia, rapidly crystallizing		6,800	
Dahlia, slowly crystallizing		7,290	
Inula helenum		6,800	
"Kahlbaum, original"	Osmometer, cellophane membrane	5 200	*Vezaby* 1951
Repeated ethanol fractionation		7,000	
Cichorium Intybus before ethanol fractionation	Osmometer, viscose membrane	3 800	Virk 1960
3rd precipitate		4 900	
12th precipitate		7,700	

4 Clearance ratios
a) inulin PEG 1 000 — 0.81 ± 0.14 — $n = 10$
b) polyfructosan-S PEG 1 000 — 0.87 ± 0.13 — $n = 10$
Comparison a) vs b) — $p \sim 0.20$
Comparison a) vs unity — $p < 0.005$
Comparison b) vs. unity — $p < 0.025$
5 Exp 332 clearance ratio — 0.87 ± 0.05 — $n = 6$
Exp 333 clearance ratio — 0.72 ± 0.07 — $n = 6$
Comparison exp 332 vs. 333 — $p < 0.001$

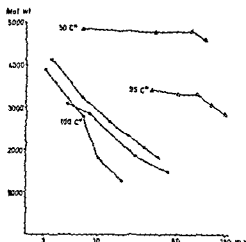


Fig 1 Depolymerization of inulin at different temperatures 100° C, ebullioscopic measurements (Drew and Haworth 1928) 95° C and 50° C, thermoelectric vapor pressure (Westfall and Landis 1936)

Commercial inulin contains both alkali stable inulin and inulin of smaller molecular weight susceptible to destruction with alkali (Cotlove 1954) The clearances of the two fractions seem to be equal in man (Walser, Davidson and Orloff 1955, Davidson and Sackner 1963)

Careless preparation of inulin infusions may depolymerize the inulin molecule and increase inhomogeneity The ebullioscopic molecular weight data of Drew and Haworth (1928) indicate rapid depolymerization by boiling (Fig 1) This might affect the inulin clearance in two ways By reduction of molecular size the filterability and clearance would increase, as seems to occur in the rat (Table I) On the other hand fructose molecules might be liberated (Vink 1950), and in the tubules these would be reabsorbed, thus lowering the measured "inulin" clearance

Certain radioactive inulin derivatives (inulin $C^{14}OOH$ and inulin OCH^3_3) pass faster than ordinary inulin through cellophane tubing in dialysis or centrifugal pressure ultrafiltration, evidently because of lower molecular weight (Chen, Terepka and Lane 1963) Inulin-$C^{14}OOH$ also passes faster than ordinary inulin from the blood into the thoracic duct lymph of the dog (Chen and Lane 1964)

The multiple sources of inulin the variation of molecular weight between different preparations or solutions, and the marked molecular inhomogeneity are severe drawbacks in the use of inulin for measuring GFR Furthermore, inulin clearance is evidently not a valid measure of GFR in the rat

Polyfructosan-S has recently been introduced as a substitute for inulin It is a fructan extracted from species of *Liliaceae* with a molecular weight around 2,756 (90 % within 2,270—4 740) (Mertz and Sarre 1963) It is readily soluble in cold water and is alkali stable In man its clearance is identical to that of inulin and in the dog its clearance is equal to that of creatinine (Harth 1963) In the rat however its clearance is slightly higher than that of inulin but still significantly below that of PEG 1 000

Polyethylene glycols offer a number of advantages as test substances They are readily soluble in cold water The chain length of the molecules can be fairly well controlled through the synthetic process thereby giving a high degree of homogeneity (Flory 1940) Mixtures of different molecular weights can be fractionated by chromatography or with trichloroacetic acid in presence of barium ions It might therefore be possible

to "titrate" the pore size in the glomeruli by using polyethylene glycols of various molecular weights

In the dog the clearances of polyethylene glycols of molecular weights from 400 up to 4,000 equal that of creatinine, whereas a preparation of molecular weight 6,000 has a 25 per cent lower clearance (Shaffer, Critchfield and Carpenter 1948) The clearances of different molecular weight PEG preparations in the rat are now being investigated

Glomerular pore size The "pore size" in the glomerular membranes may be measured by the molecular weight at which restricted permeation, i e a lower clearance, begins to occur The clearance of dextran equals GFR, with molecular weights of dextran up to 4,000 in the dog (Wallenius 1954), but up to 15 000 in man (Artursson and Wallenius 1964) Pore size" is evidently smaller in the dog than in man This is also indicated by experiments with "Levan", a polyfructan prepared from Italian rye grass, *Lolium multiflorum*, with a mean molecular weight around 5,400, but containing fractions of molecular weight towards 9,000 (Beattie and Corcoran 1952) In man its clearance approximates GFR, but in the dog its initial clearance is about 25 % below GFR

In the rat both inulin and polyfructosan-S clearances are significantly lower than the clearance of PEG 1,000, evidently because the average pore size of the glomeruli is even smaller than in the dog A relationship between glomerular pore size and size of the animal might exist within mammals

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The Effect of Dextran and Some other Colloids on the Suspension Stability of Blood from Different Species

By

R. ELIASSON and U SAMELIUS-BROBERG

Received 20 October 1964

Abstract

Eliasson, R. and U Samelius Broberg *The effect of dextran and some other colloids on the suspension stability of blood from different species.* Acta physiol scand. 1965 64 245—250 — The effect of [illegible]

The effect of dextran and other colloids on the suspension stability of washed human blood cells was extensively studied by Thorsén and Hint (1950) They demonstrated that the effect of the colloid on the ESR was a function of the concentration, molecular weight and asymmetry of the colloid Below a certain molecular weight, characteristic for each colloid, there was no decrease in the suspension stability even at very high concentration This "critical' molecular weight was about 60,000 for dextran

Although many investigations have been performed concerning the suspension stability of blood there has been relatively little attention paid to species differences

In a previous paper (Eliasson and Samelius-Broberg 1963) it was shown that a dextran preparation which clinically has proved to be a suitable plasma expander was not to be recommended in experiments on cats due to the severe decrease in the suspension stability evoked in this species Preliminary experiments revealed on the other hand, that injection of rather high molecular weight dextran ($\bar{M}_w$ about 100 000) into sheep did not cause any increase in the ESR It was therefore thought to be of interest to further study the effect of dextran on the ESR in blood from various species In some experiments the effect of other macromolecules (albumin, fibrinogen, globulin) was also studied

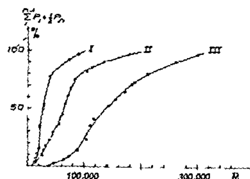


Fig 1. Molecular weight curves for 3 of the dextran fractions used
I Dextran 40,000
II Dextran 75,000
III Dextran 150,000

Material and methods

Blood was taken from healthy men and animals either through a cannula in a subcutaneous vein (man, horse, sheep), in a deep vein (urethane anesthetized rabbit), in an artery (nembutal anesthetized cat, dog and rat), or from the cut vessels of the throat immediately following death by shooting in the slaughter house

The ESR was determined after one hour according to the method of Westergren (1926) Coagulation was prevented either by mixing 4 parts of blood with one part of 0 1 M sodium citrate or by adding 0 1 ml heparin (1,000 I U /ml) per ml blood One ml of this blood was then mixed with 0 4 ml of the test solution if not otherwise is stated.

The hematocrit was determined in duplicate by a micro-method using capillary tubes which were centrifuged at 1,500 *g* for 20 min

In order to study whether an increase in the concentration of certain proteins from human plasma could affect the suspension stability of the blood, gammaglobulin or fibrinogen was

TABLE I Mean erythrocyte sedimentation rate of blood from various species without and with concentration 1 7 per cent The molecular weight distribution curve for each dextran

Species	Number of expts	Hematocrit	Controls		
			No addition	Normal saline	5 % glucose
Rabbit	4	31 (26 37)	1 (1 1)	1 (1 1)	1 (1 1)
Rat	3	23 (21 –26)	2 (1 –4)	2 (1—5)	2 (1—4)
Cat	6	30 (25 33)	4 1 10)	2 (1 –3)	2 (1 3)
Swine	4	36 34—40	5 1 12)	3 (1 6)	3 (1—5)
Man	7	33 (31 36	7 2 13	2 (1 –4)	2 (1—3)
Dog	6	31 (24 36	8 (1 25	3 (1 6)	2 (1—3)

The sedimentation rate of RBC from horse in cow plasma and of RBC from cow in horse lasma was studied by centrifuging a blood sample from one species at 1,200 g for 20 min, moving the supernatant, and resuspending the RBC in an equal volume of plasma from the ther species In these experiments heparin was used as anti-coagulant

he following colloids were used

Dextrans obtained by the fermentative action of Leuconostoc mesenteroides strain B 512 and with the average molecular weights ($\bar{M}_w$, light scattering) of about 40,000 (Rheo-

Results

A Addition of dextran

The spontaneous ESR and the effects of normal saline, glucose or equivalent volumes of various dextran preparations are presented in Table I and Fig 2 There was a great difference in the normal ESR between the species Cow and sheep had ESR < 1 mm, rat, rabbit, cat, pig, man and dog creating an intermediate group with an average ESR of 1—8 mm, while horse had a mean ESR of 78 mm

addition of different dextran fractions. Figures within brackets denote range Final dextran fraction is given in Fig 1

Dextran I in		Dextran II in		Dextran III in Normal saline
Normal saline	5 % glucose	Normal saline	5 % glucose	
1 (1—1)	1 (1—1)	16 (2—37)	6 (1—11)	128 (116—136)
1 (0—1)	1 (0—1)	10 (9—12)	4 (3—4)	102 (42—134
43 (30—50	8 (4—12)	151 (141—157)	143 (125—154)	—
3 (1—6	2 (1—3)	64 (28—111)	25 (14—45)	99 (37—136)
2 (1 6	2 (1—4)	78 (35—118)	25 (8—54)	142 (137—147)
2 (1 4	1 (1—2)	64 (12—114)	5 (1—10)	129 (66—150)

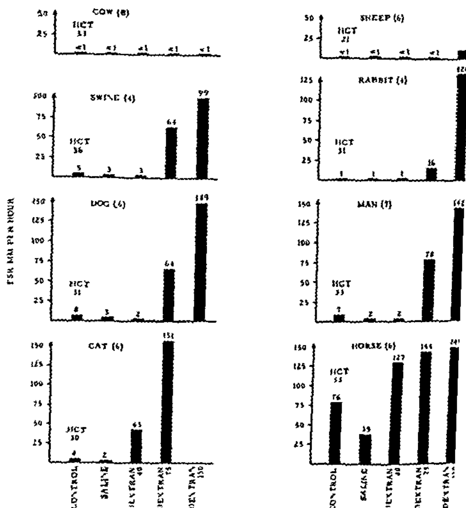


Fig 2 Normal sedimentation rate of RBC (ESR) from various species and the effect of sa and three different dextran preparations on the ESR Final dextran concentration 1 7 per c

The addition of normal saline of glucose caused a decrease in ESR probably du a dilution of aggregating proteins in plasma but possibly also to other factors Gluc decreased the ESR more than saline

The effect of the various dextran preparations was different in the various spec Dextran with a M_w of 40 000 increased the ESR of cat and horse blood but did change the ESR in the blood from the other species In all species but cow and sl dextran with M_w of 75 000 and 150,000 respectively increased the ESR but to vari degrees. The suspension stability of cow and sheep blood was not affected by any of 5 dextran fractions tested

Dextran solutions with glucose generally affected ESR to a lesser degree than those in normal saline. This is in accordance with the finding that 5 per cent dextrose decreased the ESR more than saline (see above).

B. Addition of proteins

Fibrinogen or gammaglobulin did not change the ESR of the blood from cow or sheep. The ESR of the horse blood was slightly increased by the addition of albumin (+ 1.7 %) in comparison with the addition of equal volumes of normal saline, the mean values being 73 mm as compared to 53 mm after saline and 83 mm in the control.

C. Exchange of plasma

The initial ESR of cow blood was 0 mm ($n = 2$) and in the horse blood 53 mm and 110 mm respectively. The ESR of cow RBC in the horse plasma became unchanged, i.e. zero. The ESR of horse RBC in cow plasma decreased to 32 mm and 38 mm respectively.

Discussion

A big difference in ESR between various species was observed by Hirschfeld (1907) who from experimental evidence concluded that the ESR was a function both of the aggregability of the erythrocytes and of the aggregating potency of the plasma. It is therefore possible that in a given species (e.g. man) a high ESR may be caused by changes in the erythrocytes and not only due to a changed plasma protein pattern. This was studied by Fåhraeus (1921) who concluded that in pregnant women the increased ESR was mainly due to plasma factors (for ref. see also Jeannet 1964). Experiments with human erythrocytes suspended in well-defined dextran solution may further elucidate this problem.

In cow and sheep it was not possible to induce sedimentation of the erythrocytes by adding fibrinogen, gammaglobulin or dextran. On the other hand, erythrocytes from these species can be aggregated, and an increased ESR initiated if the erythrocytes are suspended in solution of cellulose derivates, e.g. hydroxethylcellulose (Richter, personal communication). The extremely strong aggregating effect of this substance has also been reported by Hint (1962) and Richter (1963).

The post traumatic increase of various macromolecules in plasma most likely plays an important role for the development of intravascular aggregation of red cells ("sludge"). It is not clear if intravascular aggregation occurs in cow or sheep following major trauma. On the other hand, if these animals do not develop "sludge" due to a decrease in the suspension stability of the blood, this will open new ways for studying the controversial question of the relative importance of "sludge" in the complex events of changes that follow for example major traumas.

The mechanism for the increase in ESR in various diseases as well as after the infusion of certain colloids is not known (for review see Illig 1961). It has often been suggested that a change in the electrical charge of the erythrocytes would be the most likely explanation, i.e. a decrease in the negative charge would decrease the repelling forces between the red cells. This hypothesis seemed to receive further support from Bernstein *et al.* (1962) who reported that dextran with a $\bar{M}_w$ of 40,000 caused an increase in the negative charge of the erythrocytes and at the same time caused an increase in their suspension stability. More recent studies revealed, however, that dextran molecules

with strong aggregating potency also caused the same increase in electro-negativity of the red cells (Castaneda *et al.* 1964).

The present results indicate that the biological action of dextran is dependent not only on the physico-chemical properties of the dextran preparation but also on the species involved.

The authors are indebted to the AB Pharmacia, Uppsala, for the various dextran fractions and to the AB KABI, Stockholm, for the plasma proteins.

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Acta physiol. scand 1965. 64. 251—258

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Adrenergic Transmission at Vasoconstrictor Nerve Terminals Partially Depleted of Noradrenaline[1]

By

GORAN SEDVALL and JAN THORSON

Received 26 October 1964

Abstract

Sedvall G and J Thorson *Adrenergic transmission at vasoconstrictor nerve terminals partially depleted of noradrenaline Acta physiol scand* 1965 64 251—258 — The circulatory responses to activation of the vasoconstrictor nerves in skeletal muscle of the cat were studied after partial depletion of their transmitter stores by the action of reserpine and nerve impulses In spite of the presence of 30 per cent of the noradrenaline store, practically no responses to vasoconstrictor nerve stimulation were obtained provided that the physiological impulse discharge had been impinging on the stores for 2 5 hrs after reserpine administration (5 mg/kg i. v) If the impulse flow had been interrupted during this time 40 per cent of the store was present and normal vasoconstrictor responses were obtained. At 10 hrs after reserpine only a 10 per cent fraction of the noradrenaline store was left but normal responses were still produced — provided that no impulses had reached the nerve endings after the administration of reserpine About 80 per cent of this small fraction could be depleted by electrical stimulation of the vasoconstrictor nerves. No difference in the sensitivity of the blood vessels to injected noradrenaline could be found as a result of decentralization or differences in the duration of reserpine treatment. The results suggest that a large part of the noradrenaline store in vasoconstrictor nerves is not directly available for release by nerve impulses in the reserpinized cat. The ability of the nerves to transmit vasoconstrictor impulses appears to be dependent on the presence of a small noradrenaline fraction constituting 10—15 per cent of the total store

As judged from the response to nerve stimulation adrenergic transmission proceeds quite normally even when the transmitter store at the nerve endings is considerably reduced by the action of drugs (Muscholl and Vogt 1958, Rosell and Sedvall 1962 b Gaffney Chidsey and Braunwald 1963 Sedvall and Thorson 1963, Andén Magnusson and Waldeck 1964 Andén 1964) The nerve impulses, thus, do not seem to release noradrenaline from the store in proportion to its magnitude This is what we would expect if only a small fraction of the transmitter store participates directly in

[1] A preliminary report of parts of this study was presented at the Second Pharmacological Meeting Prague September 1963

the transmission process, a large proportion of the noradrenaline being unavailable for release by nerve impulses (Euler and Lishajko 1961, Trendelenburg 1961, Carlson 1964) The evidence presented for this view has so far been indirect

Results were recently obtained indicating that the noradrenaline store of the vasoconstrictor nerves in skeletal muscle is subdivided into at least 2 fractions with different sensitivity to the depletory effect of reserpine (Sedvall 1964 b) The ability of the nerves to transmit vasoconstrictor impulses seemed to be correlated to the presence of a small reserpine resistant noradrenaline fraction rather than to the magnitude of the transmitter store as a whole The results suggested the possibility of removing the different noradrenaline fractions to some extent selectively (Rosell and Sedvall 1962 a, Sedvall 1964 b)

This possibility was exploited in the present investigation, which has aimed at analyzing in more detail the extent to which different noradrenaline fractions in the vasoconstrictor nerves are available for release by nerve impulses This was accomplished by studying the circulatory responses in skeletal muscle to stimulation of the vasoconstrictor nerves after partial depletion of their transmitter stores by the action of reserpine, separately or in combination with nerve impulses

Methods

The study was made on 37 cats of both sexes weight 2 0—3 8 kg The animals were anesthetized

tor nerve stimulation and the intra-arterial injection of noradrenaline were measured as the increase in peripheral vascular resistance in per cent of the resting level The peripheral vascular resistance in the hindlimb muscles was calculated as the ratio of pressure to flow The peripheral ends of one or both transected lumbar sympathetic chains were stimulated at the level of L4 L5 with a bipolar silver electrode When both sympathetic chains were stimulated simultaneously they were placed on the same electrode Monophasic rectangular pulses (5 mS 5—15 V 10—20/sec) from a Grass Model S 4 stimulator were used Atropine (0 5 mg kg i v as sulfate) was given to prevent the action of vasodilator nerve activity Intra arterial injections of noradrenaline (0 5—5 0 μg) were given via a sidearm of the drop chamber in a volume not exceeding 0 3 ml

been expressed as free base in ng g wet weight of muscle

Results

The results of Rosell and Sedvall (1962 a) and Sedvall (1964 b) suggested that the physiological impulse flow depletes a functionally important noradrenaline fraction in the vasoconstrictor nerves of skeletal muscle within a few hours after reserpine administration This possibility was further examined in a first series of experiments Immediately

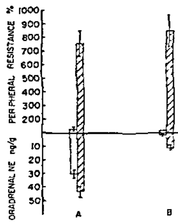


Fig 1 Noradrenaline content and peripheral vascular resistance in cat skeletal muscle following sympathetic chain stimulation 2 5 (A) and 10 hrs (B) after reserpine administration (5 mg/kg i v)
Shaded columns indicate that the sympathetic chain was transected prior to reserpine administration
Plain columns signify that the sympathetic chain was transected 5 min before stimulation
Each column is the mean of 5 expts Vertical bars indicate ± S E For full explanation see text

before the administration of reserpine (5 mg/kg i v), the flow of vasoconstrictor impulses to one hindlimb was interrupted by transecting the ipsilateral lumbar sympathetic chain The physiological impulse discharge in the vasoconstrictor nerves to the other hindlimb was allowed to continue for about 2 5 hrs after reserpine injection. The sympathetic chain also on that side was then transected A time interval of at least 5 min was allowed in order to rest the effector cells from any transmitter action, after which the peripheral ends of the two sympathetic chains were simultaneously stimulated electrically and the vasoconstrictor responses registered in the hindlimb muscles Fig 1 A presents the results from 5 cats, in which practically no response at all was obtained on the side where the impulse flow had passed uninterrupted during about 2 5 hrs of reserpine action There was still about 30 per cent of the normal noradrenaline content left in the muscles on this side The content of noradrenaline in the muscles on the other side, where the vasoconstrictor nerves had been decentralized before reserpine was administered, was only slightly higher, but the vasoconstrictor response was normal The difference in the amount of noradrenaline — which was accompanied by such an important difference in response to nerve stimulation — was only 10—15 per cent of the normal content. Similar results were obtained if the effector cells were rested for about 30 min before stimulation

In another series of 5 cats, the time interval between reserpine administration and transection of the second sympathetic chain was 10 hrs instead of 2 5 hrs Practically identical results were obtained as regards the vasoconstrictor responses, but only about 10 per cent of the normal noradrenaline content was left on the decentralized side and only about 2 per cent on the side where the nerve impulses had passed uninterrupted (Fig 1 B) The noradrenaline content of normal skeletal muscle is about 100 ng/g

The differences in vasoconstrictor responses obtained in the above experiments might have been due to a changed sensitivity of the effector cells to the released transmitter This possibility is rendered highly improbable by the results of the 2 following series of experiments These were identical to those described above except that instead of stimulating the vasoconstrictor nerves we injected noradrenaline i a into both hind-

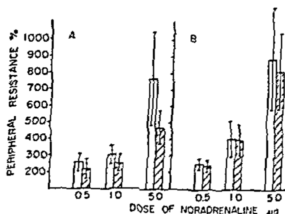


Fig 2 Peripheral vascular resistance in cat skeletal muscle following intra arterial injection of noradrenaline 2 5 (A) and 10 hrs (B) after reserpine administration (5 mg/kg i v) Shaded columns indicate that the lumbar sympathetic chain was transected before reserpine *administration* Plain columns signify that the sympathetic chain was transected 5 min before noradrenaline injection Each column is the mean of 4 expts Vertical bars represent ± S E For full explanation see *text*

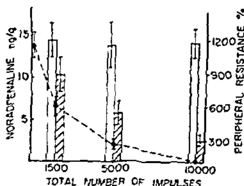


Fig 3 Noradrenaline content and peripheral vascular resistance in cat skeletal muscle following sympathetic chain stimulation after 10 hrs reserpine treatment (5 mg/kg i v) Both sympathetic chains were transected immediately before reserpine injection Shaded columns noradrenaline content, stimulated side Plain columns noradrenaline content, nonstimulated side Dotted line vasoconstrictor response to a defined test stimulus (300 imp 10 sec), stimulated side Each point and column is the mean of 5 expts Vertical bars indicate ± S E For full explanation see text

limbs The data in Fig 2 clearly show that no significant change in sensitivity to injected noradrenaline occurred as the result of decentralization or the difference in the duration of reserpine treatment

A series of experiments was deviced to investigate how far the noradrenaline fraction remaining in decentralized vasoconstrictor nerves 10 hrs after reserpine was available for release by nerve impulses Both sympathetic chains were transected before the administration of reserpine After an interval of about 9 hrs, when about 10—15 per cent of the normal noradrenaline content is still present (Fig 1), one of the sympa- [illegible] The vasocon- [illegible] red following [illegible] ts were killed after delivery of 1 500 5 000 and 10 000 stimuli respectively and the noradrenaline content in both gastrocnemius muscles determined The results can be seen in Fig 3 A definite relationship was obtained between the drop in noradrenaline content and the total number of stimuli Following the delivery of 10 000 imp, the noradrenaline content (3 ± 0 8 ng g mean S E., n 5) was highly significantly ($p < 0.001$) reduced in relation to the nonstimulated control side 14 ± 1 8) The fraction was

reduced by about 80 per cent i e to the same level as obtained when a physiological impulse flow had passed for 10 hrs (Fig 1) A similar relationship was obtained between the total number of stimuli and the reduction in vasoconstrictor responses There was always a continuous reduction in blood flow through the muscles in these animals during the stimulation period, due to the bad circulatory condition of the cat when treated for several hours with high doses of reserpine (Withrington and Zaimis 1961) The vasoconstrictions obtained in the late part of the stimulation period may thus have been slightly too small, owing to a changed pressure flow relationship (Renkin and Rosell 1962, Lewis and Mellander 1962) However, this circumstance cannot have altered the general slope of the curve, as practically no vasoconstrictor response was obtained following the delivery of 10,000 imp

Discussion

The noradrenaline content of cat skeletal muscle is localized almost exclusively in the vasoconstrictor nerves (Sedvall 1964 a, Fuxe and Sedvall 1964, 1965) The present results showed that vasoconstrictor nerve function was lost before total depletion of the transmitter stores after reserpine In spite of the presence of 30 per cent of the noradrenaline, practically no responses to vasoconstrictor nerve stimulation were obtained when the physiological impulse discharge had been impinging on the stores for 2 5 hrs after reserpine administration (Fig 1) Three different explanations seem possible for this failing response (1) a lack of available transmitter substance in the nerve endings, (2) an impaired release mechanism for the stored transmitter and/or (3) failing reactivity of the effector cells This last explanation seems highly improbable, as the blood vessels showed the same sensitivity to injected noradrenaline as those on the control side, where a normal response to nerve stimulation was obtained The same applies to the second possibility It is unlikely that reserpine *per se* blocked the release mechanism, as the latter functioned normally on the control side The explanation that the physiological impulse flow — which was presumably rather high (Iggo and Vogt 1960) — exhausted some factor responsible for the release of transmitter is also unlikely Under normal conditions, even prolonged adrenergic nerve stimulation with supra physiological frequences does not lead to fatigue of the transmission (Orias 1932, Dye 1935, Kernell and Sedvall 1964) The most reasonable explanation is that the noradrenaline fraction which was present when transmission failed is stored unavailable for release by nerve impulses

There is a possibility that the unavailable fraction is stored in the preterminal portions of the axons It has been shown, however that reserpine depletes the noradrenaline stores in different parts of the adrenergic neurone at about the same rate (Norberg and Hamberger 1964, Dahlstrom and Fuxe 1964) If the entire 30 per cent fraction was present in the preterminals, the amount of noradrenaline normally kept in this part of the neurone must be considerably larger This seems incompatible with the recent histochemical findings of Malmfors (1964) which strongly indicate that the noradrenaline in rat iris is stored predominantly in true adrenergic terminals, readily available for the action of nerve impulses It therefore seems probable that at least a large part of the unavailable fraction is present in the true terminals, but still not accessible for release by nerve impulses The fact that this transmitter fraction was not rapidly made available could indicate that mobilization of the transmitter is normally very slow However, it is also possible that reserpine blocks one of the processes which make the

preformed transmitter accessible for release by nerve impulses The findings of Euler and Lishajko (1961) and Johnson (1964) are of interest in this connection These authors presented evidence from *in vitro* and *in vivo* studies indicating that reserpine in small doses blocks the release of noradrenaline from storage granules.

The present results thus suggest that a large part of the noradrenaline in the vasoconstrictor nerves is stored unavailable for release by nerve impulses after reserpine treatment To what extent then is the transmitter available?

Considerable evidence indicates that reserpine blocks an uptake mechanism for amines in the catecholamine storing granules (Carlsson, Hillarp and Waldeck 1962, 1963, Kirshner 1962 a, b, Euler and Lishajko 1963, Hillarp and Malmfors 1964) There is a close time correlation between the inhibition of this uptake and the impairment of adrenergic nerve function in the recovery period following reserpine treatment (Lundborg 1963, Carlsson, Jonasson and Rosengren 1963, Andén, Magnusson and Waldeck 1964, Stjarne 1964) This indicates that the uptake of amines into granules is of great importance in the mechanism that makes the transmitter available for release (Carlsson 1964) No transmitter was presumably made available by uptake into granules in the present experiments, which were performed within a few hours after reserpine administration Moreover, the present results suggest that noradrenaline was not mobilized from the unavailable store In these circumstances, the ability of the nerves to release transmitter should be dependent mainly on the amount of preformed noradrenaline which is actually stored available A correlation existed between the ability of the nerves to transmit impulses and the 10—15 per cent fraction of the normal amount (Fig 1 and 3) Moreover, this fraction could be rapidly depleted by 80 per cent by stimulation of the vasoconstrictor nerves These findings suggest that in the reserpinized animal only this fraction is immediately available for release by nerve impulses This infers that the noradrenaline in the vasoconstrictor nerves is retained in at least 2 pools

It was recently shown that decentralization of the vasoconstrictor nerves in skeletal muscle disclosed a small noradrenaline fraction in the nerve terminals which disappeared at a slower rate than the main part of the transmitter store after reserpine (Sedvall 1964 b) The ability of the vasoconstrictor nerves to transmit impulses as found by Rosell and Sedvall (1962 a) appeared to be dependent on the presence of this fraction The present study supports the view that the 'available' fraction demonstrated here is identical with this 'slowly disappearing' fraction The results in Fig 1 and 3 thus indicate that practically only the 'available' fraction remained at 10 hrs after reserpine — provided that no impulses had passed Under almost identical conditions it was found that predominantly the reserpine-resistant fraction was left at this interval (Sedvall 1964 b) The evidence accordingly indicates that the 'available' noradrenaline fraction in the vasoconstrictor nerves differs from the main part of the store not only in being more directly accessible for release by the nerve impulses but also in disappearance — in the absence of impulse discharge — at a slower rate after reserpine administration These findings strongly indicate that this fraction is stored in the nerve terminal in a special compartment Future experiments must decide whether this subdivision of the transmitter store in the vasoconstrictor nerves is due to the existence of different storage mechanisms for the transmitter and/or to differences in the location of the stored transmitter in relation to the axon membrane

Quantitatively, the nerve impulse discharge plays only a minor role in catecholamine depletion after reserpine (see Sedvall 1964 b) The present results, however directly

show that the physiological nerve impulse flow plays an important role — from a functional point of view — in the mechanism of action of reserpine It seems to be responsible for the rate of depletion of the small noradrenaline fraction which mediates the transmission of impulses The rate of disappearance of adrenergic nerve function after reserpine will accordingly be highly dependent on the impulse frequency in the nerves The results presented provide a reasonable explanation for the general lack of correlation between amine levels and adrenergic nerve function following reserpine treatment and for the finding that adrenergic transmission after reserpine can be abolished before total depletion of the transmitter stores (Sedvall and Thorson 1963, Haggendal and Lindqvist 1964)

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Serpasil was generously supplied by AB Ciba Produkter, Sweden

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Absence of Monoamines in Olivo-Cochlear Fibres in Cat

By

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Abstract

[illegible]

primary catecholamines and 5 hydroxytryptamine in all probability do not act as transmitters in the efferent innervation of the organ of Corti

The course of the crossed olivo-cochlear efferent fibres was first described by Rasmussen (1946) and this has been followed by several other studies (see Rasmussen 1960, Rossi and Cortesina 1962) The uncrossed olivo-cochlear efferents were also found by Rasmussen (1960) and again described by Rossi and Cortesina (1962)

Electronmicroscopical investigations on the cochlea after transection of the crossed, or both the crossed and uncrossed, cochlear efferents (for references see Spoendlin and Gacek 1963) prove that the crossed efferents terminate on the hair cells and the afferent endings of the organ of Corti The uncrossed efferents probably end in the same region (cf Spoendlin and Gacek 1963)

Electrophysiological studies have shown the crossed efferents to be inhibitory in function (for references see Fex 1962)), as are also the uncrossed (Desmedt and LaGrutta 1963)

The cochlear efferents have a high acetylcholinesterase (AChE) activity (for references see Rossi and Cortesina 1962) and they may thus be cholinergic, releasing acetylcholine (ACh) as a transmitter In support of this view Gisselsson (1960) found an increase in the cochlear microphonics after injection of ACh into the endolymph and related these findings to the earlier results that the cochlear microphonics are increased

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by electrical stimulation of the crossed efferents (Fex 1959) The effect of such electrical stimulation has since been studied also by Desmedt and co-workers (see Desmedt 1962) However, electrophoretic application of acetylcholine into the organ of Corti as done by Katsuki (see Bullock 1964) caused suppression of the cochlear microphonics although a full report of these experiments has still to appear Furthermore, it has been shown (Desmedt and LaGrutta 1963) that the effect of cochlear efferents on the cochlear action potential and microphonics are not changed by i v injected physostigmine (an AChE-inhibitor) nor by dihydro-β-erythroidine, which blocks transmission in cholinergic junctions (Unna, Kniazuk and Greslin 1944)

The non cholinergic nature of the efferent transmitter is also suggested by the findings that subconvulsive doses of strychnine reduced or abolished the effects of electrical stimulation of the olivo-cochlear fibres (for references see Fex 1962 and Desmedt and LaGrutta 1963) The cochlear efferents are in this respect similar to the spinal interneurons that are responsible for post synaptic inhibition of motoneurones and that are in all probability non cholinergic (*cf* Curtis 1963) However, the crossed efferent inhibition is not affected by 5,7-diphenyl 1,3-diazadamantan 6-ol (1757 I S), which depresses spinal post synaptic inhibitions (*cf* Curtis 1963) Thus these spinal and the cochlear inhibitory systems may have different inhibitory transmitter substances

It has been found that the terminals of many different neuron systems in the CNS form and store monoamines (see Carlsson, Falck and Hillarp 1962, Dahlström and Fuxe 1964a) These neurons have recently been shown to be *monoaminergic* (Fuxe and Gunne 1964) The present work was made to establish whether the cochlear efferents belong to any of these specific systems The presence and direction of monoaminergic fibres can now be directly studied in the central nervous system with the use of a simple method (Dahlstrom and Fuxe 1964b) The method is based on the finding that after axotomy the respective amines rapidly accumulate in high amounts readily visualized with the fluorescence method of Falck and Hillarp in the proximal part of the fibres

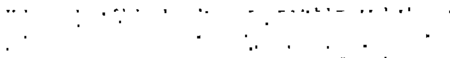
Material and methods

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In the 5 remaining cats (intraperitoneal pentobarbital 35 mg/kg) the left vestibulo-cochlear anastomosis containing the cochlear efferents was crushed with a fine forceps under aseptic conditions The technique to reach Oort s anastomosis has been described by Fex (1962) In 2 of the cats the crossed cochlear efferents (see Rasmussen 1946) were interrupted also in the brain stem when crossing at the midline just under the floor of the fourth ventricle at the [illegible]

Specimens from around the cut in the brain stem were taken in the 2 cats with such a lesion [illegible]

transversely



usually green to yellowgreen fluorescence respectively

Results

Normal animals

The organ of Corti was severely damaged in all preparations No nerve fibres with specific fluorescence were present around the few sensory cells that could be observed The stato-acoustic nerves contained a number of fine, varicose, green-fluorescent nerve fibres, some of which seemed to be related to blood vessels They were running in a part of the nerve where there are no olivo-cochlear fibres The superior olivary complex and its immediate surroundings, which would include the cells of origin of all olivo-cochlear fibres (*cf* Rasmussen 1946, 1960, Rossi and Cortesina 1962), did not contain any nerve cells with catecholamines or 5-HT

Animals with the left vestibulo-cochlear anastomosis crushed

No accumulation of catecholamines or 5-HT could be observed in the fibres above or below the lesion

Animals with section of the crossed olivo-cochlear fibres in the brain stem

A number of intensely green and yellow-fluorescent, deformed nerve fibres were observed on each side of the lesion These fibres, however, had a location and a direction different from those of the cochlear efferents (as described by Rasmussen 1946)

Discussion

The method used here to find monoamines in neurons is highly sensitive and specific (for references see Dahlstrom and Fuxe 1964a) Furthermore, axotomy of monoamine-containing nerves fibres results in a rapid and large accumulation of monoamines in the proximal part of the fibre, in the central as well as in the peripheral nervous system (Dahlström and Fuxe 1964b)

In the present work no monoamines were found in the region of the hair cells and in spite of the axotomies performed, no monoamines were found in the axons or in the soma of the cochlear efferent neurons However, certain parts of the vestibular ganglion have been found to contain CA terminals in close contact with the ganglion cells (Dahlström, Fex, Fuxe and Lennerstrand, unpublished observations) Experiments are in progress to show if they are of central or peripheral origin

It is concluded that olivo-cochlear efferent fibres do not contain NA, DA or 5-HT, hence it is unlikely that these monoamines are transmitter substances at the termination of these efferents upon the cells of the organ of Corti

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Exchangeable Water, Sodium and Chloride in the Skin of Mice

By

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Abstract

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Previous studies on the composition of connective tissue have indicated different "fluid phases" or "water compartments" Terms like "bound" or "fixed" water, "free" water and "freely moveable" water have often been applied to some of these fractions without precise definitions The various fractions of tissue water may contain electrolytes in different concentrations Electrolytes may further be stored in the tissue in a "dry" or "osmotic inactive" form, "attached" to or "bound" to various charged macromolecules

Thus for the biological function the total contents of water and electrolytes is of less interest than the part "available" to the organism in the homeostasis The available (exchangeable) part can be determined by isotope dilution methods

The purpose of the present paper has been to study the exchangeability of water, sodium and chloride in connective tissue, with special regard to the function of the acid mucopolysaccharides of the ground substance

Methods

White male mice of a single strain weighing from 22 to 28 g were used They were maintained on a standard laboratory diet with water ad libitum

Experimental groups

1) *Mice with estradiol induced edema of the skin*

On the 6th and 4th day before the experiments subcutaneous injections of estradiol monobenzoate 10 µg in 0.1 ml of arachis oil were given (*cf* Hvidberg Szporny and Langgård 1964)

2) *Mice with localized acute inflammatory edema*

On the day prior to the experiment the skin on the back was depilated by a close cut with an electric clipper followed by application of a barium sulphide depilatory After 5 min the skin was cleared of depilatory with ample amounts of water One hour before the experiment the animal was lightly anesthetized with fluothane (halothane® B P) and acute inflammatory edema induced on the naked skin by applying filter paper moistened with xylene for 2 min (*cf* Szporny *et al* 1964)

3) *Control animals* (untreated)

Experimental procedures

A) *Exchangeable water*

Using a micrometer syringe (Agla®) 50µl of a solution of ^3H_2O in 0 9 per cent NaCl containing approximately 1 µC was injected into a tail vein and the site of puncture sealed carefully Ten minutes later the animal was stunned by a neckblow, the right carotid artery was cut and 500 µl of blood sampled in a Carlsberg constriction pipette prepared with heparine saline After decapitation and bleeding the backskin was depilated as described above (animals with localized acute edema were already depilated at this stage) A particular area of the depilated skin, with underlying subcutaneous tissue was excised and divided into two pieces which were weighed on a torsion balance One sample was used for determination of the water content by freeze-drying of the sample to constant weight The other sample was used for the radioactive assay N/2 sodium hydroxide in a volume of 3 times the tissue volume (spec grav = 1 0) was added and the sample heated in a stoppered tube to 70° C for 30 min to obtain tissue destruction The proteins were precipitated by adding 10 % zinc sulphate in the same volumes as sodium hydroxide Plasma samples were treated in an identical way After centrifugation 100 µl of the clear supernatants were added to a scintillation medium described by Bray (1960) and the radioactivity measured in a liquid scintillation counter (Isotope Developments LTD) The extent of penetration of ^3H_2O into the tissue was determined by comparing the ratio of the tissue plasma radioactivity to a similar ratio for water determined chemically by freeze-drying of the tissue When the two ratios were equal penetration was complete

B) *Exchangeable sodium*

Approximately 1 µC of ^{24}Na as NaCl in 50 µl of a 0 9 per cent NaCl solution was injected i v Ten minutes later blood was sampled and the backskin depilated and excised as described for determination of exchangeable water The total skin sample was weighed on a torsion balance, put into counting tubes and the γ-radiation measured in a well type crystal connected with a "1700 scaler (Isotope Developments LTD) The radioactivity of 50 µl of blood plasma was determined simultaneously After the radioactive assay the skin samples were freeze-dried to constant weight and defattened by repeated treatments with petroleum ether and diethyl ether The dry fatfree tissue and the plasma were finally analyzed for sodium with a Beckman DU flame photometer and photomultiplier (cf Hvidberg Jensen Holm and Langgård 1963) The ratio of the tissue plasma concentrations of ^{24}Na and the same ratio for ^{24}Na determined chemically were compared If penetration of ^{24}Na into the tissue was complete the two ratios should be equal

C) *Exchangeable chloride*

50 µl of a 0 9 per cent NaCl solution containing approximately 1 µC of ^{36}Cl as NaCl was injected i v Groups of animals were examined 10 min 2 hrs or 20 hrs later Otherwise the procedures were the same as described for determination of exchangeable water The total

TABLE I The percentage of water, sodium and chloride which is exchanged in 10 minutes

	Normal skin	Skin of estradiol treated mice	Skin with acute inflammatory edema
H_2O	101±3 9 (n=11)	97±3 1 (n=12)	100±2 6 (n=11)
Na	105±2 7 (n=10)	102±4 3 (n=10)	103±3 2 (n=10)
Cl	85±1 9 (n=12)	71±2 7[1,2] (n=12)	91±1 6[1](n=10)

The values are means ± standard error

n = number of animals in each group

[1] Significantly different from 100% at $p < 0\,001$

[2] Significantly different from the corresponding control group at $p < 0\,001$

amounts of chloride in the dry fat free tissue and in the plasma were determined by automatic potentiometric titration with silver nitrate (Radiometer's® titrator) (cf Langgård, Jensen-Holm and Hvidberg 1963) in the tissue after precipitation of the proteins by Somogyi's technique (cf Cotlove 1962) The tissue plasma ratios of radioactivity and total chloride, determined chemically were compared The percentage recovery of radioactivity from skin and plasma samples were determined by adding known amounts of ^{36}Cl to skin and plasma samples. No statistically significant difference was found between the efficiency of measuring ^{36}Cl added to 10 skin samples and 10 plasma samples ($p > 0\,1$ by t test)

Results

The exchangeable part (E) of water, sodium and chloride was calculated by the formula

$$E\ (\text{per cent}) = \frac{\dfrac{\text{counts per sec per mg tissue}}{\text{counts per sec per mg plasma}}}{\dfrac{\text{total content per mg tissue}}{\text{total content per mg plasma}}} \times 100$$

The values obtained 10 min after administration of the radioactive material are shown in Table I The figures indicate that the exchange of water and sodium was completed at this time Only 85 per cent of the tissue chloride were, however, exchanged in untreated animals In estradiol treated animals the figure was as low as 71 per cent but 91 per cent in animals with localized acute inflammatory edema

The same ratio was therefore determined in normal and estradiol treated animals 2 and 20 hrs after injection of the radioactive chloride The findings are illustrated in Fig 1 Two hours after the injection 95 per cent (S E = 3 7) were exchanged in untreated mice, 82 per cent (S E = 2 0) in estradiol treated animals At 20 hrs the exchange was complete in normal skin (102 per cent ± 3 5) but not in skin of estradiol treated mice (92 per cent ± 3 0)

Discussion

The water of the skin is distributed between the intracellular and the extracellular space The extracellular space in turn, consists of an intravascular compartment and several interstitial fluid compartments The water of these compartments is held by

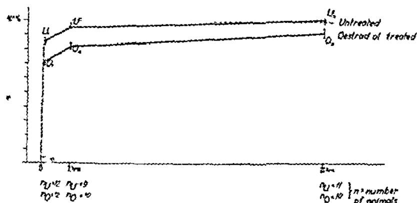


Fig 1 Exchanged chloride in per cent of the chloride content determined chemically 10 min 2 hrs and 20 hrs after injection of radioactive chloride The values for the standard error of the mean are indicated by vertical lines through the points Point O_1 is significantly different from O_2, U_1 from U_2, O_2 from O_3, O_1 from U_1, O_2 from U_2, and O_3 from 100 per cent at $p < 0.01$

forces of different natures and strength If skin is exposed to a rising mechanical pressure a certain fraction of the water can be squeezed out (Hvidberg 1959) The remaining part is held so tightly that it appears to be bound in the tissue Eilers and Labout (1946) stated that approximately half of the bound water does not serve as solvent for small ions (non electrolyte dissolving water ") This part of the tissue water may be bound to tissue proteins in a crystallized form by electrostatic forces generated by hydrophilic non polar sidegroups of the proteins on the dipoles of water (Fraser and McRae 1959 Klotz 1960) The other half which is somewhat less adherent was thought to be trapped within minute tissue cavities of the colloidal matrix (nonsaccharose dissolving water) *However the water binding capacity of connective tissue is pre* dominantly related to the hyaluronic acid (*cf* Hvidberg 1960) Kulonen (1952) points out that hyaluronic acid binds water in two essentially different ways Large amounts of water can be taken up due to the gel structure In addition small ions attached to the hyaluronic acid to the extent that they are osmotic active will attract water from the surrounding fluid Consequently another phase of fixed water adhering to the molecules of the hyaluronic acid must be counted for A freely movable fluid phase may also exist in connective tissue (McMaster and Parsons 1952)

The data of the present study (Table I) indicate that no difference exists between the exchangeability of the water molecules in these different compartments Any water molecule in the connective tissue regardless of which compartment it belongs to, regardless of which component it is bound to and regardless of the nature of the binding forces is as readily exchangeable as any other water molecule of the tissue In other words, *bound water in the sence of less exchangeable water molecules does not exist in* connective tissue The exchange of tritium does not determine the water phase only but also exchangeable hydrogen ions in proteins hyaluronic acid etc The dissociation of water at tissue pH is however inconsiderable and the exchange of hydrogen ions would therefore not influence the radioactive assay of tritiated water to a measureable extent

Similar statements can be made with regard to sodium (Table I) Calculations on the osmolarity of the tissue fluid shows that a great fraction of the cations must be present in such a way that the osmotic activity is appreciably reduced (Hvidberg Jensen Holm and Langgård 1963, Langgård *et al* 1963) The hyaluronic acid seems to be responsible for the binding of excess sodium and potassium (Kulonen 1952, Hvidberg Szporny and Langgård 1964) It is not evident whether collagen also binds sodium Kahn *et al* (1962) studying the effects of electrolytes on collagen in solution showed evidence of ion binding They further stated that it was difficult to remove ions bound to collagen although some exchange of ions took place According to Harris and Steinbach (1956) there is within isolated frog muscle a small fraction of sodium which is practically non exchangeable This non-exchangeable sodium seemed to be associated with the connective tissue rather than with the muscle fibres Manery and Bale (1941) however studying the penetration of radiosodium into the extra and intracellular phases of the tissues of rats found rapid exchange in skin kidney, liver and muscle The same authors stated that there is an essential difference between the availability of the excess sodium of connective tissue and bone In contrast with the excess sodium of bone that of connective tissue is freely exchanged with sodium in plasma

In agreement with the last mentioned authors the present study indicates that there is free exchange of sodium ions between the different compartments of the connective tissue and sodium ions in plasma

For a long time there has been a standing discussion whether chloride ions can be stored dry in the skin It is generally accepted now that the colloids of the connective tissue ground substance have amphoteric properties resulting from the coexistence of positively and negatively charged groups (Ussing *et al* 1960) Thus anions can be bound as well as cations although at physiological pH connective tissue is charac therized by a net negative charge Engel *et al* (1961) in in vivo experiments convincingly demonstrated the anion binding capacity of dermis by a potentiometric method Manery and Haege (1941) using ^{38}Cl with a short life time studied the extent and rate of penetration of chloride in different tissues They found that penetration was com pleted in skin within a few minutes In contrast with this the data of the present study strongly suggest that a certain fraction of the tissue chloride is exchanged at a much lower rate than is the main part (Table I) In skin of untreated mice this part consti tutes approximately 15 per cent of the total chloride content The findings in animals pretreated with estradiol and in animals with localized acute inflammatory edema indicate that the slowly exchangeable chloride may in some way be related to the amount of acid mucopolysaccharides of the skin Treatment with estradiol in this specific strain of mice raises the amount of acid mucopolysaccharides in the skin approximately 100 per cent without affecting the collagen content (*cf* Hvidberg Szporny and Langgård 1964) On the supposition that the slowly exchangeable chloride fraction is related to the amount of acid mucopolysaccharides treatment with estradiol should raise the percentage of slowly exchangeable chloride from the above 15 per cent to approximately 30 per cent which was actually found (Table I Skin samples with localized acute inflammatory edema per unit surface area weigh approximately the double of non inflamed skin samples *cf* Langgård *et al* 1964 and the relative amount of acid mucopolysaccharides is therefore decreased to approximately 50 per cent of the normal Thus the percentage of slowly exchangeable chloride according to

the same hypothesis, should go down from the above 15 per cent to approximately 7 5 per cent which was also found (Table I)

Of course no conclusive statement as to the nature of the slowly exchangeable chloride ions can be made on basis of the data presented here The data obtained 2 and 20 hrs after injection of the radioactive chloride (Fig 1) suggest that the slowly exchangeable chloride is exchanged at a rather constant rate

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Acta physiol. scand. 1965. 64. 269—274

From The Department of Physiological Chemistry University of Lund Sweden

The Transport Form of Free Fatty Acids in Rat Serum

A study in vitro using gel filtration

By

GORAN GORANSSON

Received 29 October 1964

Abstract

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The interaction of free fatty acid anions with albumin has been demonstrated by several authors (Teresi and Luck 1952, Goodman 1958) Using electrophoresis Laurell (1955) and Gordon (1955) have shown an interaction also between oleate and serum lipoproteins

In the present work the partition of labeled fatty acids between the serum protein and the water phases has been studied in gel filtration experiments

Materials and methods

The fatty acids used were the following

Caproic acid 1 C[14]	Obtained from The Radiochemical Centre Amersham England. Batch 4 Spec act 4 mc mM
Capric acid 1 C[14]	Obtained from The Radiochemical Centre Amersham, England Batch 9 Spec act. 4 mc mM

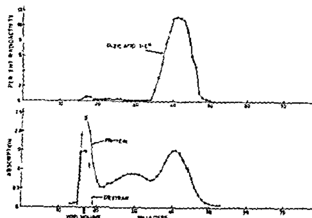


Fig 1 C^{14}-oleic acid complexed with rat serum subjected to gel filtration using Sephadex G 200 column chromatography Gel bed 60 ml Void volume 17 ml determined with dextran (m w 500 000)

Lauric acid 1-C^{14}	Obtained from The Radiochemical Centre, Amersham, England Batch 9 Spec act 21 mc/mM
Myristic acid-H^3	Nonlabeled acid obtained from the Hormel Institute Austin, Minnesota, USA The acid was labeled with H^3 as described by Bergstrom and Lindstedt (1957) Spec act 10 mc/mM
Palmitic acid 1-C^{14}	*Obtained from The Radiochemical Centre, Amersham, England* Batch 22 Spec act 4 mc/mM
Palmitic acid 9 10-H^3	Obtained from The Radiochemical Centre Amersham, England Batch 3 Spec act 330 mc/mM
Palmitoleic acid H^3	Prepared biosynthetically from palmitic acid 9 10 H^3 (see above) as described by Goransson (1965 c) Spec act 7 mc/mM
Stearic acid 9 10-H^3	Obtained from The Radiochemical Centre Amersham England Spec act app 100 mc/mM
Oleic acid 1-C^{14}	Obtained from The Radiochemical Centre Amersham, England Batch 1 Spec act 25 mc/mM
Linoleic acid 1 C^{14}	*Obtained from The Radiochemical Centre* Amersham England Batch 20 Spec act 25 mc mM
Arachidic acid 1 C^{14}	Obtained from Calbiochem California USA Spec act 2 mc mM
Oleic acid unlabeled	Obtained from Fluka AG Basel Switzerland Was used in the chemical amounts of 0 01 0 13 2 7 and 10 *µeq per* ml *of serum in* combination with labeled oleic acid to obtain an increasing mole ratio fatty acid albumin in four solutions

*Each fatty acid except caproic capric and linoleic was subjected to reversed phase chromatog*raphy and immediately before the preparation of an injection solution to liquid liquid partition as described by Borgstrom 1952 to assure that more than 99 5 % of the radioactivity was present as the respective free fatty acid No attempt was made to purify the caproic and capric acid The linoleic acid was purified by reversed phase column chromatography and $AgNO_3$ SiO_2 thin layer chromatography as described by Goransson (1965 e and the radiochemical purity was approximately 99 5 %

The amount of the sodium salt of each fatty acid given in table 1 was dissolved in 0 2 ml of ethanol and 1 ml of freshly prepared serum from normal rats added The mixture was allowed to stand at least five hours at room temperature and was then filtered The serum was usually clear (except when 10 µeq of Na-oleate was dissolved in 1 ml of serum Each solution contained palmitic acid and another fatty acid so that one C^{14} and one H^3 labeled fatty acid were present in each solution

TABLE I The partition of labeled fatty acids between protein and water phases in rat serum studied by gel filtration on Sephadex G-200 columns Gel bed 60 ml Void volume 17 ml

	µEq of fatty acid added to 1 ml of serum[1]	Percentage of radioactivity eluted with		
		13—20 ml of eluent	35—50 ml of eluent	50—70 ml of eluent
Caproic acid	0 02	0	50	50
Capric acid	0 04	1	93	6
Lauric acid	0 05	2	96	2
Myristic acid	0 05	2	98	0
Palmitic acid	0 04	4±0 8	96±0 8	0
Stearic acid	0 05	19	81	0
Arachidic acid	0 06	71	29	0
Palmitoleic acid	0 20	2	98	0
Linoleic acid	0 08	2	98	0
Oleic acid	0 01	1	99	0
Oleic acid	0 13	1	99	0
Oleic acid	2 7	30	70	0
Oleic acid	10 0	55	45	0

[1] To each solution was also added 0 04 µEq of palmitic acid so that each solution contained one C^{14} labeled and one H^{3} labeled fatty acid The values for palmitic acid are the mean ± SEM of 8 experiments whereas all the other values represent single experiments

The solutions of fatty acid in serum were subjected to Sephadex column chromatography at room temperature essentially as described by Flodin and Killander (1962) but using a gel bed [illegible]

between different fatty acids The other part was undertaken to study the effect of an increasing mole ratio fatty acid/albumin

Results

The elution pattern of rat serum proteins (see Fig 1) was similar to that obtained by Flodin and Killander (1962) for human serum proteins Three peaks were found According to the determinations of Flodin and Killander (1962 the first peak represented alfa$_2$ globulin beta$_2$ globulin alfa lipoprotein and beta lipoprotein It coincided with the void volume which equalled 17 ml as was shown in this work by the complete overlapping of the elution curves for dextran and protein See Fig 1) The second peak of protein consisted of gamma globulin and in the third peak the protein was mainly albumin

The recovery from the Sephadex columns was better than 90 % based on radioactivity measurements Comparison between columns operated at room temperature and at 37° C did not reveal any appreciable difference in the elution pattern of arachidic acid Neither was any difference noted, when the 0 2 ml of ethanol used to dissolve the fatty acid salts, was evaporated prior to the addition of the serum

Determination of the radioactivity in the eluted fractions (see Table I) showed that labeled fatty acids were eluted with the lipoprotein and the albumin fractions and in the tubes corresponding to 50—70 ml of eluent No peak of radioactivity was found in the gamma globulin fraction

In table I it can be seen that throughout the homologous series of saturated fatty acids there were certain *gradual changes in the distribution of radioactivity* The caproic acid was found both distributed over the total volume of the column and in the *albumin fraction*

The capric acid was to more than 90 % eluted with the albumin fraction The acids *from lauric to arachidic were to an increasing degree eluted with the lipoprotein fraction*

The palmitoleic, oleic and linoleic acids were to more than 98 % found in the tubes corresponding to the albumin peak

When the chemical amount of oleic acid was increased a shift in the distribution of radioactivity occurred so that more label was eluted from the column with the lipoprotein fraction

Discussion

The data in the literature dealing with the possible difference in disappearance rate of individual free fatty acids intravenously injected into animals have been somewhat contradictory Originally Fredrickson and Gordon (1958) found no differences in the disappearance rate of palmitic linoleic and oleic acid Later however Uzawa *et al* (1964) compared several fatty acids and noted that oleic acid was more rapidly extracted by the tissues than the other fatty acids Dustin et al (1961) reported that linoleic acid disappeared faster from the blood than palmitic acid Thus it now seems established that the unsaturated fatty acids with 18 carbons are more rapidly extracted than palmitic acid when injected into laboratory animals

This finding was supported by the results of Goransson and Olivecrona 1965 and Goransson (1965 a b c d and e) whose experiments also showed that long saturated fatty acids disappeared more slowly from the blood than shorter saturated fatty acids

In an attempt to add some further information to the discussion of possible differences in the turnover of individual free fatty acids in plasma the present work was undertaken The results suggested that the unsaturated fatty acids in serum were completely albumin-bound when used in amounts comparable to those injected earlier into rats The saturated fatty acids with less than 14 carbon atoms were to an increasing degree partitioned in favor of the water phase as might be expected from the decrease in hydrophobic properties of the short fatty acids The long saturated acids were to an increasing extent eluted with the lipoprotein fraction They may therefore have been complexed with the lipoproteins, but the fact that the lipoprotein fraction coincided with the void volume makes it impossible from the present data to exclude the possibility of emulsion formation However, the agreement of the present data with those of

Laurell (1955), Gordon (1955), Goodman and Shafrir (1959) and Carrol (1964) makes it probable that the fatty acids were in fact complexed with the lipoproteins in the present investigation

In any case, the present results indicate differences in the form in which the long saturated and the unsaturated fatty acids exist in the serum studied in this work This discrepancy may not necessarily be primarily determining the rate of disappearance from the blood of the fatty acids in vivo Instead the difference in disappearance and the difference in distribution between the proteins and water phases may both depend on the solubility of the individual fatty acids in water, which will be discussed below

The carbon chain of the saturated fatty acids prefers a staggered configuration in hydrophobic milieu With increasing chain length the excluded volume becomes greater This fact together with the concept that the interior of the lipoprotein molecule is less rigid than the interior of the albumin molecule makes a combination with lipoprotein more and more favorable with increasing chain length (Arvidsson 1964)

If it is assumed that the fatty acids have to pass a water phase, when transferred from the proteins in the plasma to the surface of the tissue cells and the passage through the water phase is the rate determining step in the kinetic line, the rate of uptake of the fatty acids should be dependent on the solubility of each fatty acid in water This hypothesis is supported by the data showing that the long saturated and hydrophobic fatty acids are extracted more slowly, than the short and more hydrophilic acids In the same line of observation is the faster extraction of oleic acid than stearic acid and of linoleic acid than oleic acid noted by Göransson and Olivecrona (1965) and Goransson (1965 b and c)

The finding in this work that the short fatty acids were to some extent distributed over the volume of the column is interesting in relation to other results indirectly showing that as much as 50 % of lauric acid and 90 % of capric acid fed to rats may enter the portal circulation whereas fatty acids with a chain length of more than 14 carbon atoms are transported via the thoracic duct incorporated into chylomicrons (Bloom, Chaikoff and Reinhardt 1951) Direct evidence of transport of capric acid as such in the portal blood has been presented by Borgstrom (1955) and is also in agreement with the present results

The amount of oleate dissolved in rat serum varied from 0 02 to 10 0 μeq per ml of serum in the present work This corresponds roughly to a mole ratio total fatty acid/albumin of from 2 to 22 assuming normal rat serum to contain 1 μeq of free fatty acid and 30—40 mg of albumin per ml It was found that when the total amount of free [illegible]

radioactivity was eluted with the globulins These findings agree with those of Laurell (1955) and Gordon (1955) who found that an increase of electrophoretic mobility of lipoprotein after addition of increasing amounts of oleate took place at a mole ratio fatty acid albumin equalling 3—4

This work was supported by grants from the Medical Faculty in Lund and from the U S Public Health Service (He 05302-04 MET) The excellent technical assistance of Miss Iréne Kalebsson is greatfully acknowledged

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Acta physiol. scand. 1965. 64. 275—282

From the Department of Pharmacology University of Goteborg Sweden

Energetics of Isometric and Isotonic Contraction in Isolated Vascular Smooth Muscle under Anaerobic Conditions

By

LENNART LUNDHOLM and ELLA MOHME-LUNDHOLM

Received 29 October 1964

Abstract

Lundholm L and E Mohme Lundholm *Energetics of isometric and isotonic contraction in isolated vascular smooth muscle under anaerobic conditions* Acta physiol scand 1965 64 275—282 — In experiments on isolated bovine mesenteric artery the energy production was determined from *the lactic acid production under anaerobic conditions During isometric contraction of the* [illegible] 3 to 5 fold [illegible]

stimulating and the contractile effects of adrenaline but not the effects of potassium ions

In previous experiments (Lundholm and Mohme Lundholm 1962 a, 1963) we had determined the energy metabolism during isotonic contraction of isolated vascular muscle under anaerobic conditions It was found that on contraction with catecholamines, histamine barium ions and electrical stimulation the metabolism increased during shortening of the muscle The contraction in mm and the increase of metabolism were correlated Once the muscle had attained a constant degree of contraction the metabolism ceased to increase Addition of potassium ions was followed by muscle contraction without a coincident stimulation of the lactic acid production It was nevertheless possible under certain conditions to block selectively the contractile effects of adrenaline and histamine without affecting their stimulatory action on the metabolism (Lundholm and Mohme Lundholm 1963) This somewhat tenous relation between

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Lundholm L and E Mohme-Lundholm *Energetics of isometric and isotonic contraction in isolated vascular smooth muscle under anaerobic conditions* Acta physiol scand 1965 *64* 275—282 — In experiments on isolated bovine mesenteric artery the energy production was determined from the lactic acid production under anaerobic conditions During isometric contraction of the muscle preparations addition of adrenaline or of potassium ions resulted in a 3- to 5-fold elevation of the metabolism concomitant with a rise of tension When the tension had reached maximal level the metabolic elevation was more moderate — approximately 60 per cent in the experiments with adrenaline and about 20 per cent in those with potassium ions Thus the muscle consumed more energy in attaining a certain tension level than in maintaining it Total consumption of high energy phosphate compounds on isometric contraction was estimated to exceed the preformed content by approximately 300 per cent The energy demand was appreciably greater for isometric than for isotonic contraction both during the increasing tension phase and during maintenance of constant tension — Dibenamine blocked the metabolic stimulating and the contractile effects of adrenaline but not the effects of potassium ions

In previous experiments (Lundholm and Mohme Lundholm 1962 a 1963) we had determined the energy metabolism during isotonic contraction of isolated vascular muscle under anaerobic conditions It was found that on contraction with catechol amines histamine barium ions and electrical stimulation the metabolism increased during shortening of the muscle The contraction in mm and the increase of metabolism were correlated Once the muscle had attained a constant degree of contraction the metabolism ceased to increase Addition of potassium ions was followed by muscle contraction without a coincident stimulation of the lactic acid production It was nevertheless possible under certain conditions to block selectively the contractile effects of adrenaline and histamine without affecting their stimulatory action on the metabolism Lundholm and Mohme-Lundholm 1963 This somewhat tenous relation between

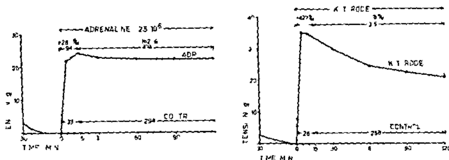
TABLE I Isolated bovine mesenteric arteries Tyrode solution with 0.5 per cent glucose in N_2. Effects of adrenaline and K ions on lactic acid production under isometric and isotonic conditions P = probability that the effect was due to chance n = number of tests t/2 = time in min to reach the half maximal value of tension or shortening t = time in min to reach the maximal value Tests with isotonic contraction from Lundholm and Mohme Lundholm (1962) After adrenaline the muscle shortened 1.9 mm (mean) and after K ions 2.0 mm (mean) The muscles were loaded with 10 g

	0—15 min lactic acid production µmol/g/15min			15—120 min lactic acid production µmol/g/105 min				
Drug contraction	control	drug	increase	control	drug	increase	t/2 min	t min
Adrenaline								
isometric	3.7 ± 0.7	10.5 ± 0.5	6.8 ± 1.0	32.7 ± 1.1	52.7 ± 2.7	20.0 ± 3.2	1.8 ± 0.2	6.4 ± 0.6
n 6			$P < 0.001$			$P < 0.01$		
isotonic	5.1 ± 1.4	6.8 ± 1.3	1.7 ± 0.6	28.9 ± 3.2	28.9 ± 3.3	0.3 ± 2.7	1.7 ± 0.1	9.2 ± 0.4
n = 7			$P < 0.05$					
K ions								
isometric	2.9 ± 0.5	12.1 ± 1.3	9.2 ± 1.2	29.8 ± 1.9	35.3 ± 1.7	5.5 ± 1.4	1.3 ± 0.14	7.8 ± 0.9
n 5			$P < 0.01$			$P < 0.02$		
isotonic	5.1 ± 1.4	2.1 ± 1.1	-2.8 ± 0.5	28.6 ± 3.0	27.4 ± 1.2	1.2 ± 3.2	0.6 ± 0.03	6.3 ± 0.6
n = 7			$P < 0.001$					

metabolic stimulation and isotonic contraction prompted us to study the metabolic behaviour during isometric contraction We elected for this purpose to investigate the effects of adrenaline — on which much of our previous research had focussed — and of potassium ions which under isotonic conditions did not stimulate the metabolism.

Methods

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cording was not absolutely isometric at a developed tension of 25 g the muscle shortened 0.5 mm. Prior to the addition of adrenaline or of potassium ions the tension was adjusted to an initial value of 2 g.

The lactic acid content of muscle preparation and suspension solution was assayed by a previously reported enzymatic method involving the use of lactic acid dehydrogenase (Lundholm, Mohme-Lundholm and Vamos 1963; Lundholm, Mohme-Lundholm and Svedmyr 1963).

experiment constituted the lactic acid production.

In the dibenamine experiments 4 of 8 preparations were first treated for 60 min with dibenamine at a concentration of 5 × 10⁻⁴ in Tyrode solution containing 0.5 per cent glucose and bubbled with nitrogen. One untreated and one dibenamine-treated preparation were then taken for determination of the initial lactic acid content, whereupon the suspension solution was changed. To one each of the three dibenamine treated preparations was added adrenaline at a concentration of 2.5 × 10⁻⁶ or K-Tyrode or NA-Tyrode containing 0.5 per cent glucose.

Results

Effects of Adrenaline and K-Tyrode on Lactic Acid Production and Tension. The results of the experiments with adrenaline are summarized in Table I and Fig. 1. During the initial 15 min period of developing tension the lactic acid production rose by approximately 300 per cent. During the next 105 min the tension remained near its maximal level and the metabolism, though significantly lower than the 15-min value, was still elevated by about 60 per cent.

TABLE II The effect of dibenamine on the stimulating action of adrenaline or K ions on lactic acid production and tension of isolated bovine mesenteric arteries Tests in N_2 and 0.5% glucose under isometric conditions Mean of 5 tests

Drug concentration g/ml	Lactic acid production µmoles/g/120 min		Maximal tension g
	Control value	Change after drug	
Adrenaline 2.5 × 10 [illegible]	31.7±2.2	+22.0±4.5 P< 0.01	27
K-Tyrode	31.7±2.2	+10.6±2.9 P< 0.05	29
Dibenamine 1 × 10 [illegible]	31.7±2.2	+2.0±5.0	0
Dibenamine 1 × 10 [illegible] + Adrenaline 2.5 × 10 [illegible]	33.7±1.0	−0.3±0.8	0
Dibenamine 1 × 10 [illegible] + K Tyrode	33.7±1.0	+7.4±2.0 P< 0.05	25

After K-Tyrode too, the lactic acid production rose substantially — by almost 500 per cent — during the first 15 min (Table I and Fig 1) For this period the maximal tension and the stimulation of lactic acid production showed a certain proportionality The quotient $\frac{\text{maximal tension in g}}{\text{lactic acid increase in mg/100 g/ 15 min}}$ was 0.44 in the adrenaline experiments and 0.43 in the experiments with K Tyrode In the latter the tension after reaching maximal level, gradually declined At 120 min the lactic acid production was elevated by only 20 per cent — a value significantly lower than that for the first 15 min

Influence of Dibenamine upon the Contractile and Metabolic Stimulating Effects of Adrenaline and K-Tyrode Since bovine mesenteric arteries contain relatively substantial amounts of noradrenaline (Schmiterlow 1948), it was conceivable that the potassium ions had elicited some of their effects by liberating noradrenaline from the vascular tissue It was found however, that dibenamine at a concentration of 5 × 10 [illegible] selectively inhibited the contractile and metabolic stimulating effects of adrenaline without influencing the corresponding effects of potassium ions (Table II), hence, it seemed likely that adrenaline and potassium ions had produced their effects via an action upon different receptors

Degree of activation in isometric and isotonic contraction In arterial muscle an isometric contraction was associated with far greater stimulation of the metabolism than was an isotonic contraction (Table I) This disparity is discussed more fully in a later section of this paper A possible explanation, however was that either a smaller number of musice fibres were activated or the degree of activation was less with isotonic than with isometric contraction *To test this hypothesis two preparations* of equal length from the same artery were contracted with adrenaline — one of them isotonically and the other

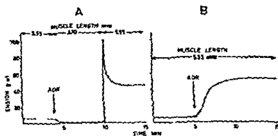


Fig 2 Mesenteric artery in Tyrode solution bubbled with N_2 0.5 % glucose Tension measured with force transducer on Grass polygraph A The tension was 6 g at an initial length of 5 55 mm The muscle was then allowed to shorten to 3 70 mm at which length the muscle gave a just detectable tension after addition of adrenaline (10^{-6}) The muscle was then rapidly (< 2 sec) elongated to 5.55 mm and the change of tension measured B The same artery at a constant length of 5 55 mm tension development after addition of adrenaline

isometrically (Fig 2) When the isotonic preparation had shortened to a constant value it was rapidly elongated to its original length i e the same length as the isometric preparation The tension of the formerly isotonic preparation increased thereby to a value two to three times that of the originally isometric preparation and then gradually declined to the same level This experiment indicated that the number of activated muscle fibres and/or the degree of activation were the same for the two preparations

Discussion

Lactic Acid Production as a Criterion of Energy Metabolism The extent to which the lactic acid production under anaerobic conditions reflects the total energy metabolism during contraction is an important question Another series of experiments was therefore conducted (Lundholm and Mohme-Lundholm 1965 a) in order to elucidate the effect of adrenaline on the high energy phosphate compound content (adenosine triphosphate and creatine phosphate) of arterial muscle during isometric contraction Seven minutes after addition of adrenaline the relevant content had fallen by an average of 0 7 μeq g and at the end of 60 min it was still depressed by 0 5 μeq/g — For each mole of lactic acid produced from glucose 1 eq of high energy phosphate compound is synthesized concomitantly — On isometric contraction in the present experiments the lactic acid production increased during the first 15 min by 6 8 μmoles g corresponding to synthesis of 6 8 μeq g high energy phosphate compounds Since as mentioned above the content fell simultaneously by 0 7 μeq g the total consumption of high energy phosphate compounds was 7 5 μeq g During the period from 15 to 120 min the lactic acid production reflected the energy metabolism fairly accurately inasmuch as the high energy phosphate compound content probably remained constant

Energy Metabolism on Isometric Contraction The arterial muscle can therefore be estimated to have consumed during isometric contraction by adrenaline approximately 7 5 μeq g high energy phosphate compounds over and above the basal metabolism. This consumption was about three times the preformed amount the latter being in the region of 2 5 μeq g Lundholm and Mohme Lundholm 1965 b A decrease in the

adenosine triphosphate and creatine phosphate contents has, as pointed out above, been demonstrated on isometric contraction The preformed high energy phosphate compound content is apparently insufficient for maximal isometric contraction of vascular muscle, such contraction depends, rather, on a continuous energy production

Time Pattern of Energy Metabolism The lactic acid production was appreciably higher during the initial phase of contraction, when tension was being produced, than subsequently when a constant tension was maintained The difference was particularly marked on contraction with K^+ ions Vascular muscle thus required a greater amount of energy for the production than for the maintenance of tension Similar findings have been reported for frog striated muscle (Hartree and Hill 1920, Hill and Woledge 1962) and for snail smooth muscle (Bozler 1930) In this respect the behaviour of various types of muscle appears to be qualitatively similar A quantitative comparison of frog muscle and vascular muscle with regard to the energy required for producing a given degree or tension will be reported in a subsequent paper (Lundholm and Mohme Lundholm 1965 a) in which the energy consumption is determined by a method that permits comparison with Hill s thermo-electric measurements It can be mentioned here, however, that the tension-production energy consumption appears to be of similar magnitude for different types of muscle, whereas the tension maintenance energy consumption is appreciably lower for vascular smooth muscle than for frog striped muscle

There could be an alternative explanation of the fact that the energy demand of vascular muscle was lower for maintenance than for production of tension. The initial elevation of tension after adrenaline and potassium ions probably reflected the degree to which the muscle was in an "active state" On the other hand, the maintenance of constant tension following the initial phase was not necessarily dependent on this "active state", but could have been due to a more passive "tonic" mechanism The experiments with K ions in which the tension fell after having reached maximal level suggested that the "active state" may change with time Further experiments were therefore conducted (Lundholm and Mohme-Lundholm 1965 b) to determine the degree of "active state" 10—120 min after addition of adrenaline or of potassium ions

With adrenaline under anaerobic conditions the "active state" remained constant throughout the experiment, but with potassium ions it decreased somewhat Assuming that stimulation of the metabolism was elicited and was commensurate with the degree of "active state", the lower energy consumption for the 15—120 min period after adrenaline could hardly have been attributable to depression of the "active state" In the potassium ion experiments, on the other hand, the reduced energy metabolism was probably ascribable in some measure to a depressed "active state" Over the period from 15 to 120 min the energy metabolism was less elevated in the potassium ion than in the adrenaline experiments The main reason the energy metabolism was lower, once the tension attained a fairly constant level, was doubtless that the muscle consumed less energy in maintaining than in producing tension

Energy Metabolism in Isotonic and Isometric Contraction of Vascular Muscle On thermo-electric determination of the energy metabolism of frog striated muscle, Hill (1938) observed that isotonic contraction developed more energy than did isometric During isotonic contraction the "heat of shortening" was added to the "heat of activation" No definite metabolic counterpart of "heat of shortening" has however, been demon-
[illegible] on (Wha-
[illegible] man and
[illegible] 1963)

Hill (1964) has shown, moreover, that the "heat of shortening" was probably overestimated in earlier studies

In arterial muscle, nevertheless, isometric contraction — as is clear from table I — was associated with far greater stimulation of the metabolism than was isotonic contraction During the first 15 min, which coincided rougthly with the initial contraction phase, the metabolic increase following adrenaline was several times greater in the isometric than in the isotonic experiments Indeed, in the former experiments but not in the latter the metabolism remained elevated even after the tension had reached a fairly constant level

On contraction of the muscle with potassium ions the lactic acid production showed no increase at all under isotonic conditions We had earlier observed that adrenaline stimulated the anaerobic lactic acid production of vascular muscle after its contractile effect had been blocked by dihydroergotamine (Lundholm and Mohme-Lundholm 1963) This lactic acid production was equal in magnitude to that associated with isotonic contraction but was substantially lower than that associated with isometric contraction The high energy phosphate compound content declined when isotonic contraction was elicited by adrenaline, but rose when the latter's contractile effect had been blocked by dihydroergotamine (Beviz and Mohme Lundholm 1965) — Under isotonic conditions, therefore, contraction is doubtless an energy-requiring process, indeed a linear relation between lactic acid production and contraction has been demonstrated It is nevertheless probable that the energy consumption of the contractile process accounted for only a part of the lactic acid production shown, for isotonic contraction, in Table I Where a correction made accordingly, however, it would add to the disparity, in terms of energy metabolism, between isotonic and isometric contractions

To what may this disparity be attributed? Since the time pattern was almost identical for the two types of contraction (see Table I) differences in duration of the contractions could scarcely have been implicated Furthermore, the respective initial lengths of the muscle preparations were practically equal The degree of activation and or the number of activated muscle fibres were probably the same for the two types of contraction (Fig 2)

One difference between frog striated muscle and vascular muscle was however that whereas the former contracted isotonically from its fully relaxed state the arterial muscle was already contracted to some extent at the outset For example, the preparations in Table I contracted isotonically only 2 mm whereas maximal contraction from the fully relaxed state may, theoretically at least amount to some 9 mm (Lundholm and Mohme Lundholm 1962 a) In these earlier experiments we also demonstrated a linear relation between the metabolic increase and the contraction in mm If assuming this relation to be a factual one and if the muscle had contracted 9 mm instead of 2 mm the lactic acid production would have been four and a half times as great, i e approximately 7 7 μmoles/g — a value somewhat exceeding that for isometric contraction *Before any final stand is taken regarding the question whether vascular muscle requires* more energy for isometric than for isotonic contraction it would be advisable therefore, to determine the energy metabolism for each type of contraction from the fully relaxed state

We are grateful to the assistance of Mr Sandor Vámós Financial support has been given by the Swedish State Medical Research Council and the Swedish National Association against Heart and Chest Diseases

TABLE IV The effect of unilateral ligation and cutting (denervation) of the sciatic nerve on the noradrenaline (NA) content of the hind leg of the cat The values are corrected for the recovery of added NA 87 per cent on the average, and for 20 per cent post mortem loss during the preparation

	Body weight (kg)	Sex	Time after denervation (days)	Difference of NA in μg between control and denervated leg
Cat 1	2 7	♀	10	14 0 } paws not estimated
Cat 2	2 5	♀	9	15 2 } paws not estimated
Cat 3	3 6	♂	60	12 5 } paws not estimated
Cat 4	3 2	♀	10	11 6 } paws not estimated
Cat 5	2 7	♀	21	14 9
			Mean ± SEM	13 6 ± 1 55

	Weight of soft tissue in g per leg Mean ± SEM	*NA content in μg per leg Mean ± SEM*
Denervated leg	202 ± 7 77 n = 5	22 4 ± 8 68
Control leg (contralateral leg)	240 ± 18 14 n = 5	36 0 ± 9 67
Normal leg (3 individual cats)	206 ± 38 69 n = 3	36 5 ± 15 60

Paws of cat 5 (soft tissue) denervated side 0 05 μg NA
control side 0 70 μg NA

Difference 0 65 μg NA

Total loss of NA after the ligation and cutting of the sciatic nerve 14 3 μg NA

in one single cm, indicating that the NA content of an axon column of 6 5 cm of the axons had been transported distally in 12 hrs giving a convection speed of 5 4 i e 5—6 mm per hr In the cat the speed was calculated to $\frac{112-9\ 2}{9\ 2 \quad 12} = 9\ 3$ mm/hr, that is 9—10 mm per hr

The accumulation distal to the site of compression has been proposed to represent signs of a transport towards the perykarya (Lubinska *et al* 1963) The amounts of NA in this part after compression was found to be very low, at most 2—3 times the normal concentrations If this distal accumulation really represents a retrograde flow, it is in all probability very low compared to the proximo distal flow

The difference in NA content found between the normal and denervated legs most probably represents the NA content of the adrenergic fibre system emanating

from the sciatic nerve Since the proximo-distal transport fills the terminals with granules at a certain rate, the corresponding number of granules must be destroyed simultaneously The average life span of the granules may thus be calculated In the rat, the terminal system supplied by the sciatic nerve contained a number of granules corresponding to 920 ng of NA In 12 hrs granules containing 15—2 = 13 ng were transported down The turn over or life-span would thus be $\frac{920}{13 \times 2}$ days = 35 days

For the cat the same calculation would give a life span of $\frac{14{,}300}{2(112-9\ 2)} = 70$ days

The figures for the life span of the granules obtained in this study (about 35 days for rat and about 70 days for cat) must of course be considered as approximate The figures, however, clearly show that the life span of the granules is of an entirely different order than the time required for renewal of the NA of the terminals which was shown to be about 1—2 days (see *i a* Bjorling and Waldeck 1965) The present study thus supports the view that the NA of the terminals is manufactured locally and that the granules renew their NA many times during their life-span

This study was supported by a P H S Research Grant from the National Institute of Neurological Diseases and Blindness (NB 05236-02 and NB 04359-03) by the Swedish Government Funds for Supporting Medical Research and the Swedish State Medical Research Council (NB 14X 166-02)

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Acta physiol scand 1966 67 289—294

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The Noradrenaline Content of the Varicosities of Sympathetic Adrenergic Nerve Terminals in the Rat

By

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Abstract

DAHLSTROM, A, J HAGGENDAL and T HOKFELT, *The noradrenaline content of the varicosities of sympathetic adrenergic nerve terminals in the rat* Acta physiol scand 1966 *67* 289—294

The noradrenaline (NA) content of the iris and vas deferens (1 cm) of the rat was determined spectrophotofluorimetrically Using the histochemical fluorescence method of Hillarp and co-workers the number of varicosities of adrenergic nerve terminals in iris and in 1 cm of vas deferens was estimated It was found that the NA content per iris and in 1 cm of vas deferens was 4.7 and 310 ng as a mean, respectively The amount of NA per varicosity was found to be about 4.2×10^{-3} pg in the iris and about 5.9×10^{-3} pg in the vas deferens The concentration of NA in the varicosities was calculated to be in the order of 0.1—0.3 per cent and the number of amine granules in a varicosity to be in the order of 1 500

As demonstrated by Euler (1956) the transmitter substance of the peripheral adrenergic nervous system is noradrenaline (NA) which is stored in the terminals of the sympathetic nerves By means of methylene blue staining Hillarp (1946) was able to elucidate the structural organization of the peripheral autonomic nervous system The terminal parts consist of long slender processes on which at regular intervals widenings appear to form numerous small terminal bags The same picture was obtained when it became possible to visualize the adrenergic transmitter by means of the histochemical fluorescence method of Hillarp and coworkers The terminals were seen as long convoluted bands of intensely fluorescent dots separated by interparts of a low or no fluorescence These fluorescent dots represent the varicosities (the presynaptic bags where the transmitter seems to be located in very high concentration They are separated by thin fibre segments containing very low amounts of NA (Norberg and Hamberger 1964 Malmfors 1965

The purpose of the present investigation was to determine the approximate amount of NA in each varicosity

TABLE I The noradrenal ne (NA) content in ng in rat iris

Number of irides per estimat on	Rec very in per cent of added amount of NA (50 to 100 ng)	NA per ir s The values corrected for the recovery
13	50	4 8
15	8	4 4
15	72	4 4
10		6 1
15		4 5
15	77	3 9
16		4 0
Mean value ± SEM		4 6 ± 0 28

The add t on of NA performed to samples conta n ng 1 to 2 irides

Material and Methods

1964 and Norberg and Hamberger 1964)

B ochem stry Ir Ir des of normal rats were dissected out under d ssect on m croscope Ze ss) on ... hment ceps the ary bod I as a much darker colour because of its large dens ty of vessels The d ssec on t me for each ir s was about 3 5 m n Ten to 16 r des were collected n each sample Seven samples were exam ned (Table I) To sam ples conta n ng 1 2 r des a known am ount of NA was added before the homo gen zat on to check he recovery of tl e est mat on procedure

Vas deferens Only the m ddle 15 20 mm of the vas hav ng an even d ameter as used The m ddle part f the vas was taken out and all vessels and membranes were rem oved When the vas had contracted max m ally after the d ssection 10 m m p eces were carefully measured and cut out Ten samples were aken each conta n ng 1—2 p eces To 2 of the samples a known am ount of NA was added before the homogen zat n to check tl e recovery dur ng the est mat on procedures

The r s and the vas deferens samples were collected in 5 ml ice cooled 0 4 N perchlo c ac d contain ng 20 mg of d sod um-ethy lened amin e te raacetate (EDTA ar d glass homogen zed NA was determ ned fluorimetr cally accord ng to Haggendal (1963

... a r and ... ons are ... I st nctly ... graphed from the per phery to the centre each at 16 places ch sen from all over the prepara on The prep-arat ons used were carefully examined all over n the m croscope to con r I tl e even s re ch of the iris and the homogenous d str but on of the term nals A m cr scale (Object m cr meter 1/100 mm, E Leitz Wetzlar was m crophotographed a the same me as he sect ons o cl eck the magn f cat on of the m croscope Then the photograpl s were counte l for var cos t es over a known surface 0 187 mm² n all m cropl otograpl s s ud ed Tl e total surf ce of ea h of the s retch prepared ir ses used was 13 3 mm 39 mm

... ng the ... ormal ... aga n ... d 4 μ to check the shr nkage dur ng t e procedures ... Care was taken to cut the cross sect ons at a r gl t angle to the thick long tud nal and cross sections

TABLE II The noradrenaline (NA) content in μg per cm rat vas deferens. One individual vas deferens per sample

The recovery in per cent of added NA (0.50 to 1.00 μg)[1]	NA amount per cm. The values corrected for the recovery
77	0.32
	0.29
	0.35
	0.25
	0.27
	0.35
89	0.33
	0.22
	0.32
	0.37
Mean value ± SEM	0.31 ± 0.015

[1] The addition of NA performed to samples containing 1 to 2 mm vas deferens

longitudinal axis and likewise to cut the longitudinal sections through the middle axis. The thickness of the sections was chequed by means of interference microscopy (Johansson 1957) using a microscope newly developed by Jungner Optics. The sections were mounted in immersion oil and immediately microphotographed together with a microscale (see above). The counting was performed on microphotographs from both longitudinal and cross sections in 250 and 410 × magnifications. Corrections for double counting were performed according to the principle of Billingsley and Ranson (1918). Since the form of the varicosities was found to be slightly ovoid (1.5 μ × 2.5 μ) the correction factors were calculated separately for longitudinal sections and for cross sections. Longitudinal sections. The number of varicosities throughout the entire thickness of the muscle layer of a certain length was counted and the number of varicosities per volume unit of muscle tissue was determined. The total muscle volume of a 10 mm long piece of vas deferens was determined as the difference between the volumes of two cylinders, the radius of the larger one reaching to the periphery of the muscle layer and the radius of the smaller one to the inner surface of the muscle layer. The radii were determined as a mean of ten random transverse sections.

Cross sections. The number of fluorescent dots of all intensities was counted within a sector, the angle varying between 12° and 42°. The total number of visible varicosities in the whole section was then calculated.

Results

As seen from Table I the NA content per iris was found to be 4.6 ± 0.28 ng. The number of varicosities in one iris was calculated to be about 1.1×10^6. The amount of NA per varicosity would thus be about $\frac{4.6}{1.1 \times 10^6} = 4.2 \times 10^{-3}$ pg. Only the strongly fluorescent, true varicosities were counted and thus not the preterminal axons with their scarce irregularities which have a very low to low fluorescence intensity. The varicosity diameter varied about 1.5—2 μ, measured in the microphotographs, see also Malmfors 1965. Assuming a spherical shape the volumes thus varied about 1.8—4.2 μ^3, which would give a concentration of NA of about 0.1—0.23 per cent or 1 000—2 300 μg/g wet weight, assuming the specific weight of the varicosity to be about 1.

The NA content in 10 mm of vas deferens was found to be 310 ± 15 ng (Table II). The total number of varicosities in this piece was found to be about 52.5×10^6, taken as a mean of the values obtained at countings in 5 longitudinal sections (3 of 4 μ and 2 of 2 μ) and 10 cross sections (6 of 4 μ and 4 of 2 μ). The mean value for the longitudinal sections was calculated to be 53×10^6 varicosities and for the cross sections to be 52×10^6. Each varicosity in the vas deferens thus seemed to contain $\frac{310}{52.5 \times 10^6} = 5.9 \times 10^{-6}$ pg of NA. The varicosity size — and also the fluorescence intensity — was observed to be about the same as in the iris, the diameter varying between 1.5—2.5 μ, giving volumes about 1.8—8.1 μ^3 and a NA concentration of 0.07—0.32 per cent.

Discussion

Several facts support the reliability of the data for the NA contents in the tissues examined. 1) The spectrophotofluorimetrical method used for the biochemical determinations of NA is of high sensitivity (see i.a. Häggendal 1963). 2) The specimens were dissected out within 5 min. after killing the animals (minimal postmortal destruction). 3) The recovery of added NA was good and corrected for.

The histochemical fluorescence method used for the counting of the varicosities is highly specific and sensitive (see discussions in e.g. Dahlström and Fuxe 1964 and Norberg and Hamberger 1964). Under optimal reaction conditions (Hamberger, Malmfors and Sachs 1965) less than 10 μg/g of NA can be detected in the microscope in 10 μ thick sections (Norberg and Hamberger 1964). Thus, there is no doubt that the intensely fluorescent dots observed represent the adrenergic varicosities, and that the very weakly fluorescent parts between the varicosities contain very low amounts of NA (about 10 μg/g compared to that of the varicosities (Norberg and Hamberger 1964, Malmfors 1965). The varicosities can also be easily separated from the weakly fluorescent irregularities often seen in the preterminal axon i.a. in the iris (Malmfors 1965). Furthermore, the presence in the vas deferens preparations of small pieces of sectioned varicosities would be observed in the microscope used, since the NA concentration in the varicosities was found to be very high.

The counting of the varicosities performed as described above gives in all probability a good evaluation of the number of varicosities. Since the iris preparations are stretch-preparations and easy to count for intensely fluorescent dots, the obtained figure may be regarded as close to reality.

For the vas deferens the procedure was more complicated. However, attempts were made in order to make the calculations as reliable as possible. 1) Control measuring of the original 10 mm piece revealed a shrinkage of 5—6 per cent, and corrections for this were done. 2) The thickness of the sections from the ultro-tome was chequed on about 20 separate sections, 10 of which were used for counting. The thickness was found to vary within a certain range (see Material and Methods) and the mean values were used for the calculations. 3) Every fluorescent spot was counted, even those having the weakest fluorescence intensity, and double counting

of the varicosities was always corrected for (see Material and Methods) 4) Two different section thicknesses were used for the countings The mean values obtained from the two types of sections had a good correlation 5) Both longitudinal sections and cross sections were counted Also between these two kinds of sections a good correlation was obtained (see Results) 6) For calculating the size of the muscle column in 10 mm of vas deferens, the outer and inner radii were determined as a mean of 10 random measurements in 10 microphotographs of separate cross sections Thus, the obtained figure of 52 5 $\times$ 10^6 varicosities in a 10 mm piece is in all probability representative

The figure of the NA content in the varicosities in the vas deferens (5 9 $\times$ 10^{-3} pg) was found to be somewhat higher than the corresponding figure for the iris varicosities (4 2 $\times$ 10^{-3} pg) However, since the counting procedure was very different in the two tissues, the results obtained may be considered to show a good correlation The discrepancy of the two figures may either be due to errors in the counting procedures which are not possible to control, or to the fact that the varicosities of the vas deferens normally may contain higher NA concentrations than those of the iris

The figure obtained in this study of about 5 $\times$ 10^{-3} pg of NA per varicosity gives a concentration in the varicosity cytoplasm of about 0,1—0,3 per cent, or 1,000—3,000 μg/g wet weight These figures are somewhat lower that those suggested by Norberg and Hamberger (10,000 μg/g wet weight, 1964) obtained mainly on the basis of the fluorescence intensity of the varicosities Their figure is more close to the concentrat on figure of NA for cow suprarenal granules obtained by Hillarp and Nilsson (43,000 μg/g wet weight, 1954)

Assuming a diameter of the amine storage granules of 500 Å and presuming that the amine concentration in the nerve granules is the same as in the adrenal medullary granules (4,3 per cent, Hillarp and Nilsson, 1954) the number of storage granules in a varicosity with a volume of *e g* 2 μ^3 and an amine concentration of 0 2 per cent may be calculated to be about 1,500 This figure may, however, vary considerably with *e g* the size of the varicosity, and is based on the assumption that the major part of the NA in the varicosity is stored within granules

The interference microscopical measurements of the Araldite® sections were kindly made by engineer Anund Bäck Jungner Optics to whom we wish to express our gratitude

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Acta physiol scand 1966. 67. 295—299

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Uptake of 3,4-Dihydroxyphenylalanine and 5-Hydroxytryptophan by Catecholamine Forming *Mast Cells in the Hamster*

By

J ADAMS RAY, A DAHLSTRÖM and CH SACHS

Abstract

ADAMS RAY, J, A DAHLSTRÖM and CH SACHS *Uptake of 3 4 dihydroxyphenylalanine and 5 hydroxytryptophan by catecholamine forming mast cells in the hamster* Acta physiol scand 1966 67 295—299

The uptake of L-DOPA D DOPA and 5 HTP in the CA forming mast cells in the hamster was studied by means of the histochemical fluorescence method of Hillarp and coworkers It was found that the mast cells of the cheek pouch did not develop any fluorescence even after high doses of L-DOPA [illegible]

to a large degree the development of fluorescence after L DOPA treatment MAO inhibition did not increase the fluorescence intensity in normal animals whereas pretreatment with the MAO inhibitor nialamide was necessary to give an obvious yellow fluorescence in the mast cell granules after 5 HTP administration It is suggested that these mast cells contain a true DOPA decarboxylase with a certain stereospecificity for the L isomer The presence of a weak MAO activity in the cells is also discussed

From a biochemical point of view the tissue mast cells includes at least two distinctly different cell types in all mammalian species so far examined with adequate methods (the guinea pig may be an exception) (Adams Ray *et al* 1964) One of these types constitutes local tissue stores of catecholamines (CA) or 5 hydroxytryptamine (5 HT) With the help of the sensitive histochemical fluorescence method of Hillarp and coworkers for the demonstration of monoamines at cellular and subcellular levels strong evidence has been obtained that in the hamster and rabbit (and probably also the cat) one group of mast cells which seems to be localized predominantly to the skin, contains a primary CA Adams Ray *et al* 1965) The experiments further seemed to show that these mast cells have the characteristics of CA forming cells, which can readily take up L 3 4 dihydroxyphenylalanine (L-DOPA) but not the

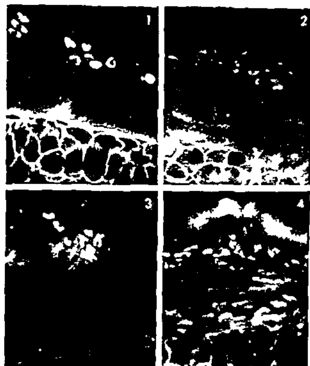


Fig 1 CA mast cells in the ear of a L-DOPA treated hamster (10 mg/kg) 1 hr before the killing The strong fluorescence intensity in the cytoplasm of the cells is seen At the bottom is seen a part of the ear cartilage × 195

Fig 2 CA mast cells in a hamster treated with D DOPA (50 mg/kg) 1 hr before death Note the weak fluorescence intensity of the cytoplasm compared to the strong intensity of the mast cells in Fig 1 × 195

Fig 3 CA mast cells with a very strong fluorescence intensity developed after the administration of L-DOPA (50 mg/kg) 1 hr before death × 195

Fig 4 CA mast cells in the ear of a hamster pretreated with the decarboxylase inhibitor NSD 1015 (100 mg/kg) 1/2 hr before the administration of L DOPA (50 mg/kg) 1 hr before the killing Compare the medium fluorescence intensity in these cells to the very strong intensity in the cells of Fig 3 The increased back ground fluorescence in this animal is in all probability due to presence in the tissue of the precursor amino acid × 195

D isomer, contain DOPA decarboxylase and have a specific amine storage mechanism that is blocked by reserpine Observations made in the present study have given further strong support to this view

Material and Methods

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DOPA and nialamide were dissolved as described by Dahlstrom and Fuxe (1964) The other substances used were dissolved in 0 9 per cent NaCl under gentle warming Only freshly made solutions were used

... method (Hillarp and co workers have recently
... der the
... 5 HTP)
... ro-beta
... ence in
... 1965)
... he fluorescence microscope used (Corrodi and Hillarp 1963 ... ... 1965) it is thus possible to directly visualize uptake and accumulation of very small amounts of the amino acids and amines in the mast cells

TABLE I Changes in the fluorescence of the CA mast cells in the hamster ear skin following treatment of the animals with drugs interfering with monoamine metabolism The +symbols represent average fluorescence intensities among the cells. Dopamine (calculated as free base) and the amino acids were given subcutaneously NSD 1015 was administered intraperitoneally 1 hour before DOPA — Number of animals within brackets

Treatment (Number of animals within parenthesis)		Dose (mg/kg)	Time before killing (hrs)	Fluorescence
Untreated control				(+) to + green
L-DOPA	(4)	10	1	2 + green
L-DOPA	(8)	50	1 to 24	4 + green
D DOPA	(4)	10	1	As untreated controls
D DOPA	(6)	50	1	(+) to 2 + green
L-DOPA[1]	(3)	10	1	2 + green
L DOPA[1]	(2)	50	$^1/_2$	3 + to 4 + green
L-Tryptophan	(2)	50 and 100	$^1/_2$ and 1	As untreated controls
L-Tryptophan[1]	(4)	100	$^1/_2$ and 1	As untreated controls
DL-5 HTP	(3)	50 and 100	$^1/_2$ and 1	As untreated controls
DL-5 HTP[1]	(3)	50	$^1/_2$ and 1	As untreated controls
DL-5 HTP[1]	(5)	100	1	+ to 2 + green yellow to yellow
Dopamine[1]	(3)	2 × 25 (with an interval of $^1/_2$ hr)	$^1/_2$	As untreated controls
NSD 1015 }		100		
L-DOPA }		50	$^1/_2$ (4)	Weakly increased
			1 (4)	+ to 2 + green
Nialamide	(4)	100	$4^1/_2$	As untreated controls

[1] The animals were pretreated with nialamide (100 mg/kg p 4 hrs)

Results

Mast cells in the ear skin

Most of the abundant mast cells in the ear skin of untreated hamsters develop a distinct but weak green fluorescence after the histochemical treatment There is strong evidence that this fluorescence is due to the presence of low amounts of a primary CA (Adams Ray *et al* 1964) in all probability DA (Adams Ray, *et al* 1965) These cells will in the following be called CA mast cells A small part of the skin mast cells belong to the same category as the cheek pouch cells and do not show any specific fluorescence even after the administration of a large dose of L-DOPA

The CA mast cells exhibited a markedly increased green fluorescence after the administration of a low dose 10 mg kg) of L DOPA (Fig 1) After a high dose (see Table I) they were strongly fluorescent after 30 min and remained so during the following 24 hrs Similar findings were made in animals pretreated with nialamide This potent monoamine oxidase (MAO) inhibitor did not induce

vious increase in the fluorescence when administered alone No significant increase was observed after administration of two large doses of DA in nialamide pretreated animals

An increased green fluorescence was observed also after a large dose of D-DOPA but the increase was clearly lower than that obtained after a 5 times lower dose of the L-isomer (compare Fig 1 and 2)

The CA mast cells showed a normal appearance in animals treated with L-tryptophan No obvious changes in their fluorescence were found following the administration of 50 or 100 mg/kg of DL-5-HTP to untreated animals If the animals had been pretreated with nialamide, however, a distinct but weak increase of the fluorescence intensity was observed after the largest dose The fluorescence had also changed from green to yellow and the fluorescent material was clearly localized to the mast cell granules

Pretreatment with the potent DOPA decarboxylase inhibitor NSD 1015 (see Carlsson 1964) prevented to a large extent the increase in fluorescence observed after a large dose of L-DOPA

Mast cells in the cheek pouch

The abundant mast cells in the cheek pouch all belong to a category which is distinctly different from the CA mast cells in the skin Confirming the results obtained previously they did not show any fluorescence in untreated animals and did not become fluorescent even after a large dose of L-DOPA Nor was any of the mast cells in this region observed to develop any fluorescence in the animals treated with DOPA, tryptophan or 5 HTP according to Table I

Discussion

The present results on the CA mast cells of the hamster ear strongly support and extend the results of previous investigations (Adams Ray *et al* 1964 1965) By far the majority of the mast cells in the hamster ear have been shown to contain a primary CA, probably DA They also from DA from DOPA due to the presence of DOPA decarboxylase activity in these cells since very little CA was formed if pretreatment with a DOPA decarboxylase inhibitor had been performed Furthermore, DA was not taken up by the cells to any significant degree (see also Adams Ray *et al* 1964) The uptake and or decarboxylation of L DOPA by the mast cells seems to be fairly stereospecific, since much less CA was formed if D DOPA was given Since the animals were not eviscerated, however, the small increase in the amine contents of the mast cells observed after D DOPA administration may be due to an uptake and transformation by the viscera of D DOPA into L-DOPA The stereospecificity observed supports the view that a true DOPA decarboxylase is present, since this enzyme is known to be stereospecific *cf* Holtz 1960

The DA formed was retained and stored in high concentrations in the cytoplasmic granules of the mast cells The amine storage mechanism of these granules has been shown to be blocked by reserpine (Adams-Ray *et al* 1964)

The experiments with L tryptophan and 5 HTP with or without pretreatment with the potent MAO inhibitor nialamide seem to demonstrate that the uptake and/or decarboxylation of L DOPA is fairly specific for this amino-acid

Only after high doses of 5 HTP and in combination with MAO inhibition it was possible to observe a yellow fluorescence in the cytoplasmic granules This yellow fluorescence was in all probability due to the formation and storage of 5 HT in these granules Furthermore, these experiments show that some MAO activity probably exists in these mast cells The activity is however, in all probability very low, since nialamide was not able to potentiate the increase in fluorescence intensity observed after L DOPA

Thus there is evidence that the enzymes DOPA decarboxylase and MAO exist in the CA mast cells of the hamster ear and that the capacity of these cells to take up and/or decarboxylase L-DOPA is fairly specific However, no dopamine β oxidase activity seems to be present (Adams Ray *et al* 1965), since no increase in the noradrenaline content has been found biochemically in the hamster ear even 24 hrs after L DOPA administration, at which time the mast cells were still strongly fluorescent

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For a generous supply of nialamide we are indebted to the Swedish Pfizer Stockholm Sweden

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Changes in Rat Pineal Stores of 5-Hydroxytryptamine after Inhibition of its Synthesis or Break-Down

By

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Abstract

FALCK, B, CH OWMAN and E ROSENGREN *Changes in rat pineal stores of 5-hydroxytryptamine after inhibition of its synthesis or break down* Acta physiol scand 1966 67 300—305

causes parenchymal 5 HT to disappear within one to two hours while little change is found in the nervous indole level This indicates a turn-over rate for 5 HT in the pinealocytes considerably

The concentration of 5-hydroxytryptamine (5-HT) is higher in the pinal gland than in any other organ of the rat Up to 70—100 ng per gland have been reported (Bertler *et al* 1963, 1964, Quay 1963, Snyder *et al* 1965) The amine is distributed equally between the parenchymal cells and the sympathetic nerve fibres (Bertler *et al* 1963, 1964) Although these fibres are noradrenergic, the intrapineal portions also contain 5-HT, which originates from the parenchymal cells (Owman 1964 a and b) The nerves arise in the superior cervical ganglia (Owman 1964 a) and form a dense plexus of terminal fibres around the intrapineal vessels and the pinealocytes (Ariëns Kappers 1960, Owman 1964 b, Håkanson and Owman 1965)

During the normal day-and-night photoperiod, pineal 5-HT exhibits a very marked circadian rhythm from a maximum around noon, to a minimum about 90 per cent lower near mid night (Quay 1963) Considering the above-mentioned distribution of 5-HT between pineal nerves and parenchyma the rhythm apparently involves both these storage sites

5 HT in the rat pineal is obviously synthetized by the pinealocytes, which have a very high activity of the decarboxylating enzyme (Snyder and Axelrod 1964, Håkanson and Owman 1965) The gland also contains hydroxyindole 0 methvl transferase (Wurtman *et al* 1963, Quay 1964) and monoamine oxidase in large quantities (Wurtman *et al* 1963, Håkanson and Owman 1965) leading to the forma tion of melatonin and tryptophols as well as acid metabolites from pineal 5-HT (see Quay 1965)

The aim of the present investigation was to elucidate changes in pineal 5 HT concentration with both histochemical and chemical methods, after inhibition of synthesis and break down of the 5 HT with various drugs under daylight conditions Moreover, it turned out that the experiments also allowed an estimation of the turn over rate of 5 HT in the pineal gland of the rat The significance of the sympathetic system in this pineal 5 HT metabolism was also investigated

Material and Methods

Experimental All experiments were performed or microscop c experiments compound H 22/54 enzymatic hydroxylation of tryptophan (Carls dose of 400 mg/kg i p to 13 animals killed 1

90 min

In one group of seven animals the monoamine oxidase inhib tor nialamide (Niamid Pfizer) was given alone in a dose of 300 mg/kg i p and the animals killed after 3 hrs Another group (11 an mals) was pretreated with nialamide before NSD 1015 or Ro 4 4602 (doses and times as above)

B lateral cervical sympathectomy was performed in 5 animals by removing both superior cerv cal ganglia under light ether anesthesia Five days after operation NSD 1015 or Ro 4 4602 in the afore mentioned doses was injected

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All injections were given in a single dose A corresponding volume of saline was injected to 6 control animals which were compared with 32 untreated controls

The number of animals given above does not include those used in the fluorimetric determina tions this appears in the section on *Spectrophotofluorimetric assay* and Fig 2

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fluorescence microscopical conditions used The techn cal performance followed the descript on by Falck and Owman (1965)

The mean values obtained for 5 HT in the tissues in the various experiments were compared using the Student s t test

Results

Fluorescence microscopy of the normal pineal gland of the rat, whether untreated or injected with saline, revealed a rich plexus of delicate varicose nerve fibres (Fig 1 a) emitting an intense yellow light due to the content of 5 HT (*cf* Bertler *et al* 1964) Although the intrapineal nerves also seem to contain a small amount of noradrenaline this amine is not directly visible owing to the masking effect of the intense fluorescence of the 5 HT in the fibres (Bertler *et al* 1964, Owman 1964 a) The pineal nerves enclose both the vessels and the parenchymal cells, the latter also emit a yellow 5 HT fluorescence of high intensity (Fig 1 a) Accordingly, large amounts of 5 HT is detected fluorimetrically in the gland (Fig 2) Administration of the monoamine oxidase inhibitor, nialamide, produced no or only a slight increase in the fluorescence of the intrapineal nerve fibres No change was noted in the parenchymal fluorescence Chemically, the 5 HT content of the pineal gland was not significantly increased while that of the brain was increased about threefold ($P < 0.001$) (Fig 2)

Treatment of the animals with the decarboxylase inhibitor Ro 4 4602 (Fig 1 b) caused at most a slight if any, decrease in the yellow fluorescence of the pineal nerves However a complete or in a few cases almost complete disappearance of the parenchymal fluorescence occurred The result was the same after administrating NSD 1015 (Fig 1 c) or H 22/54 (Fig 1 d) Chemical determinations revealed about 80 per cent loss of pineal 5 HT in both the Ro 4 4602 and NSD 1015 experiments ($P < 0.01$) No significant changes were registered in brain 5 HT in the 2 expts (Fig 2) A similar loss of parenchymal 5 HT could still be demonstrated histochemically by Ro 4 4602 and NSD 1015 after pretreatment with nialamide as well as after bilateral cervical sympathectomy — which as shown before (Bertler *et al* 1963 caused the fluorescence in the pineal nerves to disappear Administration of pyrogallol did not cause any change in the fluorescence intensity of either pineal sympathetic nerves or parenchymal cells

Compared with the fluorescence microscopic picture seen in the synthesis inhibition experiments the depleting agent Ro 4 1284 had the converse effect the fluorescence in the nerves had disappeared while no or only a slight decrease occurred in intensity of the yellow light emitted by the parenchymal cells

Discussion

In the pineal gland of normal rats the 5 HT content is equally distributed between the sympathetic nerve fibres and the parenchymal cells (Bertler *et al* 1964) After the administration of NSD 1015 or Ro 4 4602 both of which strongly inhibit the decarboxylating enzyme present in large amounts in the rat pineal gland (Håkanson and Owman 1965) a considerable proportion (80 per cent) of 5 HT rapidly disappears from the gland With reference to the present fluorescence microscopic findings and to the above mentioned calculated distribution of the pineal 5 HT this seems to imply a near total loss of the amine in the pinealocytes while nervous



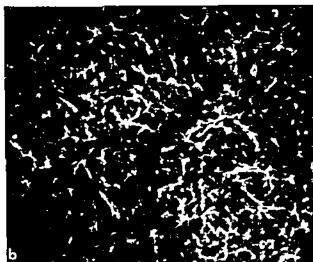


Fig 1 Fluorescence photomicrographs of rat pineal gland Magnification 100 ×
(a) Normal animal Yellow 5 HT fluorescence of high intensity in sympathetic nerve fibres and parenchyma
(b) Ro 4-4602 No change in fluorescence intensity of pineal nerves while the parenchyma appears essentially dark The rich autonomic ground plexus is well visible after elimination of the masking effect of the parenchymal fluorescence

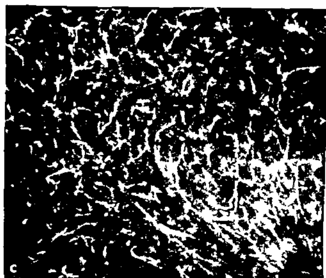




Fig 1 *(contd)*
(c) NSD 1015 (d) H 22/54 Same result as after treatment w th Ro 4 4602 (F g 1b)

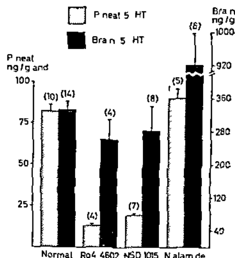


Fig 2 Fluorimetric determination of 5 HT in pineal (nanograms per pineal gland) and whole brain (nanograms per gram tissue) Number of samples within brackets

5 HT is lowered by about 30 per cent There are several reasons arguing for the assumption that the changes are due to an enzyme inhibition, rather than to *e g* an amine depletion mechanism (a) The drugs cause little or no decrease in brain 5 HT, as shown in the present and earlier (Pletscher and Gey 1963, Carlsson 1964) studies Moreover, Ro 4-4602 does not alter the spontaneous release of C^{14} noradrenaline in heart (Burkard *et al* 1964) (b) The changes in the pineal stores of 5 HT following treatment of the animals with agents known to have 5 HT depleting effect such as Ro 4 1284 (Pletscher and Gey 1963) or reserpine, differ widely from those occurring after treatment with NSD 1015 or Ro 4 4602 (see also Bertler *et al* 1963, 1964) (c) Compound H 22/54, which inhibits the hydroxylation step in the bio synthesis of 5 HT, and which differs in molecular structure from the hydrazines NSD 1015 and Ro 4-4602 (Carlsson *et al* 1963, Corrodi 1965), produces changes in pineal 5 HT stores very similar to those seen after administration of the latter drugs It is assumed (Carlsson *et al* 1963, Corrodi 1965) that H 22/54 is able to lower the brain amine levels owing to interference with the step thought to be rate limiting in the synthesis of neuronal monoamines Since H 22/54 also inhibits catechol-0 methyl transferase as demonstrated in the above mentioned investigations, it was thought that it might inhibit pineal hydroxyindole 0 methyl transferase too and interfere with the microscopic interpretation of the synthesis inhibition This was attempted to rule out by comparing the effects of another catechol 0 methyl transferase inhibitor, pyrogallol *cf* Ross and Haljasmaa 1964 a and b) This latter substance did not alter the normal fluorescence microscopic appearance of the rat pineal gland

The findings therefore indicate a very rapid turn-over of 5 HT in the pinealocytes Such a rapid pineal 5 HT metabolism apparently occurs even during the phase in the circadian rhythm when there is a prominent build up of the 5 HT content in the gland see Quay 1963) An intact sympathetic innervation does not seem to be essential for the turn over rate o

The results further suggest that the resistance of the pineal cells to reserpine, as discussed by Owman (1964 b), cannot be due to a low amine release, though it might be in the adrenal medullary cells (*cf* Carlsson 1965)

In accordance with the findings by Quay and Halevy (1962), no significant increase occurred in pineal 5 HT after treatment with a monoamine oxidase inhibitor, even though the inhibitor presently used — nialamide — severely interferes with pineal monoamine oxidase (Håkanson and Owman 1965) The neuronal 5 HT in brain, on the other hand, showed an almost threefold rise It seems likely that monoamine oxidase, present in large quantities in the pineal gland, is an important physiologic pathway for the break down of 5 HT within the gland (Quay 1964) The failure of nialamide to increase pineal 5 HT as well as to interfere overtly with the turnover rate of the indole may therefore be due to an overflow' from the pineal cells, augmented metabolism of 5 HT along other available pathways (Weissbach *et al* 1960 Axelrod and Weissbach 1960, McIsaac *et al* 1964), or a decreased synthesis due to the inhibition of pineal decarboxylase produced by nialamide (Håkanson and Owman 1965)

It should be pointed out that 5 HT is probably secreted as such by the pineal cells since the 5 HT present in the intrapineal nerves is obviously taken up from the pinealocytes with displacement of noradrenaline (Owman 1964 a) An equilibrium between the nervous and parenchymal pools of 5 HT may therefore explain the estimated 30 per cent decrease of 5 HT in the pineal nerves concomitant with the loss of parenchymal 5 HT upon treatment with the decarboxylase inhibitors

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TABLE I Distribution of dopamine in the brains of normal and reserpine-treated rats

		No pretreatment		Reserpine 10 mg/kg i p, 24 hrs	
Brain part		μg/g	μg/side	μg/g	μg/side
Pons+medulla oblongata		0 09	0 010	0 01	0 001
		0 07	0 007	0 02	0 002
		0 03	0 003	0 02	0 001
	Mean	0 06	0 007	0 02	0 001
Mesencephalon		0 25	0 018	0 07	0 004
		0 23	0 013	0 06	0 004
		0 21	0 015	0 08	0 006
	Mean	0 23	0 015	0 07	0 005
Prosencephalon		1 3	0 61	0 14	0 08
		1 1	0.52	0 11	0 06
		1 1	0 58	0 06	0 03
	Mean	1 2	0 57	0 10	0 05
Corpus striatum		6 5	0 43		
		7 1	0 44		
		6 6	0 40		
	Mean	6 7	0 42		
Tuberculum olfactorium+ +Nucl accumbens+Septum+ +Nucl interstit striae terminalis		1.2	0 08		
		1 4	0 07		
		1 2	0 08		
	Mean	1 3	0 08		
Rest of the prosencephalon		0 06	0 02		
		0 04	0 01		
		0.08	0 03		
	Mean	0 06	0 02		

Material and methods

Adult male Spra[illegible]

neostriatum and the other parts of the prosencephalon from 6 rats were separated The DA of the above mentioned parts was determined spectrophotofluorometrically after cation exchange

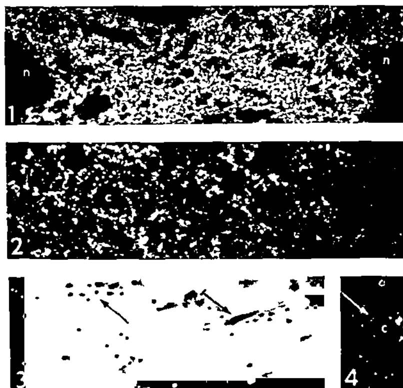
also cell bodies with very low DA concentrations were counted two animals were treated with nialamide (500 mg/kg i p 4 hrs before sacrifice) plus L-3 4 dihydroxyphenylalanine (L-DOPA 100 mg/kg s c 1 hr before sacrifice)

Distinct varicosities of the neostriatal DA nerve terminals were obtained by incubating thin slices of the neostriatum from 3 rats in a medium containing α methyl noradrenaline in a concentration of 1 μg/ml (Hamberger and Masuoka 1965) After freeze-drying (Thieme 1965) and formaldehyde gas treatment 2 μ thick Araldite sections were made by means of an LKB ultrotome (Hökfelt 1965) The thickness of the sections was checked as elsewhere described (Dahlström Häggendal and Hökfelt 1966) The varicosities were counted on microphotographs magnified 500 or 3 000 x Double counting was avoided as mentioned above The total volume of the neostriatum was calculated from an atlas of the rat brain (König and Kl ppel 1963) and so was also the volume of the fibrae capsulae internae The total number of nerve cell bodies in the neostriatum was determ ned in toluidine blue stained paraffine sections (10 μ) by counting the number of cell bod es with distinct nucleoli in a certain volume in various parts of the nuc caudatus putamen Correction for double counting was made (Billingsley and Ranson 1918—1919)

Results and discussion

Cell bodies The density of the DA nerve terminals seems to be rather low and about the same in the mesencephalon and in the pons plus the medulla oblongata (Fuxe 1965) Therefore the difference found between the DA concentration of the mesencephalon and of the pons plus the medulla oblongata (Table I) may be due to the the presence of DA in many nerve cell bodies in the mesencephalon but not in the pons plus the medulla oblongata (*cf* Dahlström and Fuxe 1964) If this assumption is correct the amount of DA in these cell bodies on one side would be about 11,000 pg (1 pg = 10^{-12} g) However, not all of the DA nerve cell bodies in the mesencephalon belong to the nigro neostriatal neurons About 80 % of the prosencephalic DA was located in the neostriatum and most of the rest was found in the tuberculum olfactorium the nucleus accumbens and the dorso lateral part of the nucleus interstitialis striae terminalis (Table 1, *cf* Fuxe 1965) If the proportion between the DA contents of the cell bodies and the terminals is similar in the different neuron systems, the substantia nigra would contain 8 800 pg DA per side The substantia nigra occupies about 10 % of the rat mesencephalon (König and Klippel 1963) The DA concentration of this nucleus would therefore be approximately 1 4 μg/g This level is somewhat higher than those found in the human substantia nigra (Bertler 1961 0 40 μg/g Hornykiewicz 1963 0 46 μg/g) It is to be noted however that the DA level in the corpus striatum in those experiments was more than 50 % lower than in the rat corpus striatum, probably mainly due to a postmortal destruction of DA Therefore the values of Bertler and Hornykiewicz appear to agree satisfactorily with the present results

The total number of DA nerve cell bodies in the substantia nigra on one side was 3,500 on the average, which was found also in the animals treated with nialamide-L DOPA Thus there would be $\frac{8\,800}{3{,}500} \approx 2\ 5$ pg DA per cell body The diameter of the DA cell bodies varied mainly between 25 and 35 μ If the cell bodies are regarded as spheres the DA concentration in them can be calculated to be $\frac{2\ 5 \times 10^{-12}}{\frac{4}{3}\pi \times 15^{3} \times 10^{-12}} \approx$



200 μg/g wet weight This concentration of DA in models gives about the same fluorescence intensity as the cell bodies have (Hillarp, unpublished data)

It cannot be excluded, however that a larger part of the mesencephalic DA than calculated above was present in nerve terminals In favour of such an assumption may be the facts that there was a reduction of the DA level in the mesencephalon 24 hrs after reserpine 10 mg/kg i p (Table I) and that there is a marked recovery of the fluorescence in the cell bodies at this interval (Dahlstrom and Fuxe 1964) With the histochemical technique used however it may be difficult to decide whether normal levels have been reached or not in the DA cell bodies 24 hrs after this dose of reserpine since the normal fluorescence is rather intense But if the recovery is complete the DA content of one cell body would be as low as 0 8 pg and the DA concentration about 60 μg g

Nerve terminals The DA content of the neostriatum on one side was 420,000 pg on the average (Table I There were about 3 500 nigro-neostriatal DA neurons on each side since this number of DA nerve cells was found in the substantia nigra

Therefore the terminal parts of an average neuron would contain $\frac{420\,000}{3{,}500} = 120$ pg DA. In the calculations given above, 2.5 (or 0.8) pg DA per cell body was obtained. Thus the amount of DA would be approximately 50 (or 150) times larger in the terminals than in the cell bodies.

The density of the DA terminals in the neostriatum was very high. For that reason in the 10 μ thick sections usually employed the terminals often cover each other and a diffuse green fluorescence is obtained. However, in sections as thin as 2 μ which were used in the present study it has been possible to visualize the individual, very fine to fine DA terminals of the neostriatum (Fig. 1—4) and to count their varicosities. The mean total number of varicosities of the neostriatum on one side was 1.7×10^9. Thus there would be $\frac{1.7 \times 10^9}{3\,500} \approx 500\,000$ varicosities per neuron. The volume of all the DA varicosities would be $1.7 \times 10^9 \times \frac{4}{3}\pi \times 1.5^3 \times 10^{-12} \approx 0.025$ mm^3 if the diameter is 0.3 μ or $1.7 \times 10^9 \times \frac{4}{3}\pi \times 3.5^3 \times 10^{-12} \approx 0.3$ mm^3, if it is 0.7 μ. If the total volume of the DA varicosities is assumed to be 0.10 mm^3 the DA varicosities should occupy $\frac{0.10}{33} \times 100 \approx 0.3\,\%$ of the neostriatal volume. The mean total number of nerve cell bodies in the neostriatum was about 4×10^6, i.e. there were $\frac{1.7 \times 10^9}{4 \times 10^6} \approx 400$ DA varicosities per cell body and its processes on the average.

One varicosity can be calculated to contain $\frac{120}{500\,000} \approx 2.5 \times 10^{-4}$ pg DA on the average. The varicosities can be seen also when the DA content has been reduced by about 90 %, e.g. 1 hr after an i.p. injection of 10 mg/kg reserpine (Fuxe 1965, Andén 1966). These findings would imply that it is possible to detect $\frac{2.5 \times 10^{-17} \times 6.06 \times 10^{23}}{153} \approx 100\,000$ molecules of DA with the histochemical technique used.

The diameter of the DA varicosities in the neostriatum varied mainly between 0.3 and 0.7 μ in diameter. The mean DA concentration of a spherical varicosity 0.4 μ in diameter can thus be calculated to be $\frac{2.5 \times 10^{-16}}{\frac{4}{3}\pi \times 2^3 \times 10^{-15}} \approx 8\,000\ \mu g/g$ wet weight. The fluorescence intensity was about the same as that obtained with this concentration of DA in a model system (Hillarp unpublished data). Previously Carlsson *et al.* (1964) have estimated the NA concentration in the varicosities of the spinal cord to about 10 000 μg/g. In this connection it is of interest to point out

that in the adrenal medulla (rat) which may be considered as an analogue of the monoamine nerve terminal the catecholamine concentration is about 10,000 $\mu g/g$ (Carlsson and Hillarp 1956, Hillarp, Jonsson and Thieme 1959) A peripheral, sympathetic, adrenergic neuron system has recently been the object of a similar quantitative study on its transmitter, noradrenaline (Dahlstrom and Haggendal 1966, Dahlstrom, Haggendal and Hokfelt 1966) The distribution of noradrenaline is very much the same in such neurons as that of DA in the nigro neostriatal neurons

In a length of 7 7 μ the DA terminals generally contained 5 to 7 varicosities Thus, the total length of all branches of the terminal system of one neuron would be between $\frac{500{,}000 \times 7\,7}{5} \times 10^{-4} = 77$ cm and $\frac{500{,}000 \times 7\,7}{7} \times 10^{-4} = 55$ cm The average total volume of the neostriatum was 33 mm^3, *i e* all the DA nerve terminals occurred in a nucleus with a thickness not exceeding 0 5 cm Therefore the DA terminals of an indivudual neuron probably innervate many cells in the neostriatum This divergence of the DA nerve terminals must be of great functional importance (*cf* Hillarp 1946, 1959 concerning the organization of the innervation apparatus of the peripheral, autonomic nervous system)

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Ascending Monoamine Neurons to the Telencephalon and Diencephalon

By

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Abstract

ANDEN, N-E, A DAHLSTROM, K FUXE, K LARSSON, L OLSON and U UNGERSTEDT. *Ascending monoamine neurons to the telencephalon and diencephalon* Acta physiol scand. 1966 *67*. 313—326

By means of sensitive and specific methods for histochemical and biochemical determination of dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) we have succeeded in demonstrating and mapping out a number of ascending monoamine neuron systems from the lower brain stem [illegible] vating e g the limbic forebrain structures, the neocortex and the hypothalamus, 4) ascending 5 HT neurons with cell bodies situated mainly in the raphe nuclei of the mesencephalon (nuc raphe dorsalis nuc raphe medianus), and axons running uncrossed mainly in the medial forebrain bundle innervating e g the limbic forebrain structures and the hypothalamus. The effects observed on the amine levels of the neurons represent intraneuronal and not transsynaptic changes

By means of the histochemical fluorescence method of Hillarp and Falck for the cellular demonstration of dopamine (DA), noradrenaline (NA) and 5 hydroxytryptamine (5-HT) the existence and distribution of catecholamine (CA) and 5-HT nerve cell groups and of CA and 5-HT nerve terminals in the central nervous system have been demonstrated (Carlsson, Falck and Hillarp 1962, Dahlström and Fuxe 1964, 1965, Fuxe 1965) However, in intact and untreated animals it is not possible to determine to which monoamine nerve cell bodies the various monoamine terminal systems belong, since the non terminal parts of the axons have amine concentrations too low to be visualized with the present technique One way to solve this problem is to increase the amine levels of the entire neuron by means of drugs For example, after monoamine oxidase (MAO) inhibition the level of 5-HT is increased so much

that the whole 5-HT neuron can be visualized under the fluorescence microscope (Dahlström and Fuxe 1964, 1965) Another way is to make lesions in the various monoamine neuron systems and study the amine changes in the parts proximal and distal to the lesion The latter method has been used in a series of investigations in our laboratories and preliminary reports have been published (Anden *et al* 1964 a, Andén *et al* 1965 a, b, Anden *et al* 1966 a) In the present paper a more comprehensive account of these studies is given In addition the effects of some other types of lesions are described

Experimental

[illegible] About 500 animals [illegible]

or halothane anesthesia *In some of the animals instead of electrolytic or electrothermic lesions* the neostriatum was removed by suction under ether anesthesia (Andén *et al* 1965 a) After operation the animals were kept at a temperature of 29° C. If needed they were given post-operative care. The rats were killed by decapitation 1 to 60 days after the operations Most lesions were unilateral and therefore it was possible to detect crossing of the pathways The main lesion types are shown in fig 1

Histochemistry The medulla oblongata pons mesencephalon diencephalon and telencephalon were dissected out, freeze-dried treated with formaldehyde gas embedded sectioned mounted and examined under the fluorescence microscope as previously described in detail (Dahlstrom and Fuxe 1964 Hamberger Malmfors and Sachs 1965) The exact locations of the lesions were determined under the fluorescence microscope and/or when the sections had been stained with toluidine blue under the light microscope

Biochemi [illegible] analyzed for DA NA and 5-HT [illegible] arlsson and Roser [illegible] r 1961 Andén an [illegible] in the various forebrain structures was determined as described by Andén Magnusson and Rosengren (1965)

Results

The nigro neostriatal DA neuron system

I Lesions of the substantia nigra

A clear correlation was found between the extent of the destruction of the DA nerve cell bodies in the substantia nigra and the reduction of the number of DA nerve terminals or the amount of DA in the neostriatum (nuc caudatus putamen) as revealed histochemically and biochemically respectively After unilateral cell body damage (Fig 1, D) the changes in the neostriatum occurred only on the operated side The DA content of the telencephalon plus diencephalon was reduced by about two thirds at most (for details, see Anden *et al* 1964 a) Since the neostriatum contains about 80 % of the brain DA the loss of this amine mainly reflects changes in this nucleus

II Lesions at the level of the corpus mamillare

The lesions usually involved the crus cerebri, the lateral part of the lateral hypothalamic area and parts of the Forel s field H_1 and field H_2 and of the zona incerta

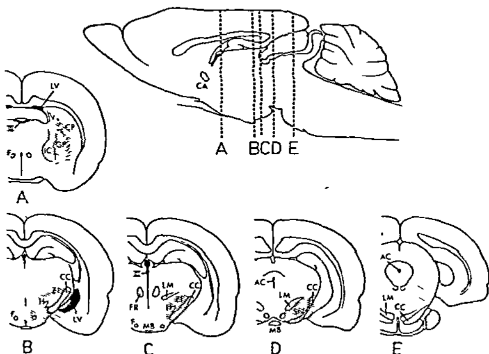


Fig 1 Pro ect on d [illegible]

corpus mamillare, SN substantia nigra ZI zona incerta, III third ventricle

(Fig 1, B, C) A reduction in the number of DA terminals and in the amount of DA in the neostriatum on the operated, but not on the unoperated side, was always observed After lesions involving the lateral part of the rostral crus cerebri, the decrease of DA nerve terminals occurred mainly in the lateral part of the neostriatum When, on the other hand, the medial part of the rostral crus cerebri was damaged the decrease of DA nerve terminals occurred mainly in the medial part of the neostriatum Very large lesions which also involved part of the medial forebrain bundle caused an almost complete disappearance of the DA nerve terminals and DA of the neostriatum Thus, still at this level a large part of the DA nerve fibres have not yet entered the crus cerebri In a large number of the DA nerve cells in the substantia nigra, retrograde cell body changes were observed After 2—4 days the cells were swollen and contained an increased concentration of DA whereas after 3—4 weeks the cells were shrunken exhibited chromatolysis and had a reduced DA concentration These findings were seen especially after very large lesions involving the medial forebrain bundle (Andén *et al* 1965 b The DA and NA levels of the telencephalon

TABLE I. The concentrations (μg/g tissue) of dopamine (DA) and noradrenaline (NA) in the rat telencephalon plus diencephalon after lesions of the nigro-neostriatal DA neurons on the left side at the level of the corpus mamillare or of the globus pallidus. The figures in brackets refer to number of experiments. The values are means ± SEM

		No lesion	Lesion at the corpus mamillare	Lesion at the globus pallidus
DA	right side	1.15 ± 0.076 (6)	1.18 ± 0.044 (30)	1.32 ± 0.056 (8)
	left side	1.27 ± 0.028 (6)	0.46 ± 0.062 (30)	0.52 ± 0.048 (8)
Difference		+11%	−61% (P < 0.001)	−61% (P < 0.001)
NA	right side	0.48 ± 0.026 (6)	0.55 ± 0.025 (30)	0.46 ± 0.027 (8)
	left side	0.50 ± 0.027 (6)	0.43 ± 0.032 (30)	0.44 ± 0.049 (8)
Difference		+4%	−22% (P < 0.01)	−4% (N.S.)

TABLE II. The L-3,4-dihydroxyphenylalanine decarboxylase activity in the various structures of the rat telencephalon and diencephalon after lesions of the nigro-neostriatal dopamine neurons on the left side at the level of the corpus mamillare. The activity is expressed as the formation of dopamine in μg/g tissue during 45 min incubation with L-3,4-dihydroxyphenylalanine. The figures in brackets refer to number of experiments. The values are means ± SEM

Brain region		Control group	Lesion group	Difference between the sides in the lesion group
Hippocampus	right side	78 ± 16 (3)	71 ± 8.5 (6)	−3%
	left side	67 ± 10.5 (3)	69 ± 11.4 (6)	(N.S.)
Thalamus	right side	120 ± 2 (3)	160 ± 12 (6)	−15%
	left side	130 ± 8 (3)	140 ± 11 (6)	(N.S.)
Amygdala	right side	69 ± 11.6 (3)	69 ± 6.8 (5)	−19%
	left side	66 ± 9.7 (3)	56 ± 9.6 (5)	(N.S.)
Hypothalamus	right side	160 ± 27 (3)	180 ± 26 (6)	+2%
	left side	160 ± 12 (3)	190 ± 40 (6)	(N.S.)
Corpus striatum	right side	310 ± 31 (3)	350 ± 25 (11)	−67%
	left side	340 ± 31 (3)	115 ± 14 (11)	(P < 0.001)
Septum + Tuberculum olfactorium + Nucl. accumbens + Nucl. interstitialis striae terminalis	right side	180 ± 8 (3)	180 ± 19 (6)	−28%
	left side	200 ± 19 (3)	130 ± 17 (6)	(N.S.)
Neopallium	right side	150 ± 30 (3)	149 ± 17 (6)	−18%
	left side	150 ± 12 (3)	110 ± 20 (6)	(N.S.)

plus the diencephalon as well as the L-3,4 dihydroxyphenylalanine decarboxylase activity of the various forebrain regions after these lesions are presented in Table I and II, respectively

III Lesions at the level of the globus pallidus

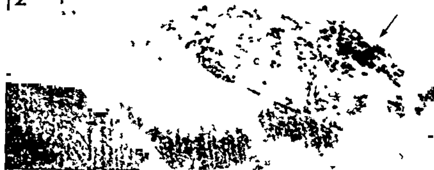
These lesions usually involved both the lateral part of the internal capsule and the globus pallidus (Fig 1, A) A marked reduction in the number of DA nerve terminals was observed in the lateral and ventromedial parts of the neostriatum The dorsomedial part of the caput, however, appeared to have a normal number of DA nerve terminals, indicating that the fibres to this region ascend in the medial part of the internal capsule If only the globus pallidus was damaged changes were less marked and occurred mainly in the area lateral to the globus pallidus The DA and NA levels of the telencephalon plus the diencephalon after this type of lesion are given in Table I

IV Removal of the neostriatum

Most of the DA nerve cell bodies within the substantia nigra — but no other monoamine cell bodies — showed increased fluorescence and were somewhat swollen 2—4 days after the removal of the neostriatum After 3—4 weeks they showed marked decrease in fluorescence together with a reduction in the cell volume and a loss of the stainability of the Nissl substance Simultaneously with the increased fluorescence in the cell bodies a rapid and marked accumulation of DA was observed within very abundant swollen nerve fibres in the internal capsule It was even possible to trace these fibres caudally in the retrolenticular part of the internal capsule down to the most rostral part of the crus cerebri (Andén *et al* 1965 a) Recently an even more complete picture of the distribution of the DA fibres was obtained after very extensive destruction of the neostriatum It was found that, after leaving the substantia nigra area most of the nigro-neostriatal DA fibres become aggregated in a bundle which ascends just medial and dorso-medial to the ventral part of the crus cerebri (Fig 2) At the level of the posterior part of the median eminence the bundle turns into the ventral rostral part of the crus cerebri and enters and diverges into the retrolenticular part of the internal capsule Running rostrally and dorsally in the internal capsule the fibres then ascend in the fibrae capsulae internae to innervate the neostriatum This DA neuron system seems to be involved in motor functions, since a blockade of the DA transmission in the neostriatum causes *e g* rigidity (Andén *et al* 1966 b)

Other ascending DA pathways

The DA fibres innervating the tuberculum olfactorium, the nuc accumbens and the dorsolateral part of the nuc interstitialis striae terminalis probably ascend somewhat medially to those innervating the neostriatum This is assumed from the findings that the DA terminals and the DA of the former areas disappeared on the operated side after lesions involving the medial forebrain bundle, but not after lesions restricted


2


3


4

to the crus cerebri Most of these fibres seem to originate from the CA cell group surrounding the cranial half of the nuc interpeduncularis (group A 10 according to Dahlström and Fuxe 1964), since cell body changes occurred in this group after lesions in the medial forebrain bundle (Andén *et al* 1965 b) Furthermore, recent data on the effect of destruction of a large part of group A 10 showed a disappearance of the DA nerve terminals and DA in the tuberculum olfactorium and the nuc accumbens Recently it has also been found that very large lesions involving the internal capsule and the lateral part of the medial forebrain bundle at the level of the globus pallidus markedly reduced the DA levels of the neostriatum, the nuc accumbens and the tuberculum olfactorium without significantly affecting the NA levels of the telencephalon and diencephalon After lesions in the medial forebrain bundle at the level of the anterior commissure the DA nerve terminals and the DA of the tuberculum olfactorium and the nuc accumbens, but not of the neostriatum, disappeared In conclusion it can be stated that the DA fibre bundles to the tuberculum olfactorium and the nuc accumbens arise from CA cell bodies of group A 10 and ascend medially to the crus cerebri into the medial forebrain bundle, most of them running laterally to the NA nerve fibres and ventromedially to the nigro-neostriatal DA nerve fibres

NA pathways

Lesions in the medial forebrain bundle (Andén *et al* 1965 b) or in the latero-caudal part of the tegmentum in the mesencephalon (Fig 1, E and Fig 3) (Andén *et al* 1966 a) caused a loss of at most 75 % of the NA in the telencephalon and diencephalon on the operated side (Table III) indicating that at least 75 per cent of the ascending NA fibres originate in cell bodies of the pons and the medulla oblongata After these lesions there was a disappearance of most or all of the NA nerve terminals on the operated side in the hypothalamus (*e g* nuc dorsomedialis hypothalami (Fig 4), nuc paraventricularis, nuc periventricularis hypothalami), the preoptic area, the ventral part of the nuc interstitialis striae terminalis, the septal area, the amygdaloid complex, the hippocampal formation, the gyrus cinguli (Fig 5) and the neocortex The fibres to the neocortex ascend from the medial forebrain bundle dorsally into the tractus diagonalis and then turn caudally into the superficial parts of the white substance This can be observed a few days after very extensive removal of the neocortex Following the caudal mesencephalic lesions retrograde cell body changes

Fig 2 Strongly green fluorescent nigro-neostriatal DA nerve fibres at the level of the posterior part of the med [illegible]
C [illegible]
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Figure) in the tegmental ret cular m dbra n (mainly the dorsal part lateral and inferior to the periaqueductal gray and dorsal to the med al lemn scus at the level of the inferior collicle The aqueduct is seen [illegible]
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TABLE III The concentrations (µg/g tissue) of 5 hydroxytryptamine (5 HT) noradrenaline (NA) and dopamine (DA) in the rat telencephalon plus diencephalon after lesions on the left side in the medial forebrain bundle at the level of the corpus striatum or in the latero-caudal part of the mesencephalic formatio reticularis The figures in brackets refer to number of experiments The values are means ± SEM The animals in the two columns to the left weighed 300—400 g while those in the two ones to the right weighed about 190 g

		No lesion	Lesion at the corpus mamillare	No lesion	Lesion in the latero-caudal mesencephalon
5 HT	right side	0 55±0 024 (3)	0 57±0 035 (18)	0 35 (1)	0 34 (1)
	left side	0 52±0 005 (3)	0 26±0 024 (18)	0 29 (1)	0 37 (1)
Difference		−5%	−55% (P < 0 001)	−17%	+9%
NA	right side	0 48±0 026 (6)	0 60±0 025 (21)	0 37±0 019 (5)	0 39±0 023 (10)
	left side	0 50±0 027 (6)	0 33±0 030 (21)	0 38±0 021 (5)	0 13±0 010 (10)
Difference		+4%	−45% (P < 0 001)	+3%	−67% (P < 0 001)
DA	right side	1 15±0 076 (6)	1 13±0 047 (17)	0 71±0 064 (5)	0 80±0 094 (5)
	left side	1 27±0 028 (6)	0 49±0 096 (17)	0 73±0 068 (5)	0 72±0 077 (5)
Difference		+11%	−57% (P < 0 001)	+3%	−10% (N S)

Furthermore, this finding means that the cells of group A 1 give rise, not only to descending bulbo-spinal NA fibres (Carlsson *et al* 1964, Dahlstrom and Fuxe 1965), but also to ascending NA fibres innervating the diencephalon and telencephalon Those 25 per cent of the NA nerve terminals in the telencephalon which remain after the caudal mesencephalic lesions may possibly arise from axons originating from group A 10 and/or A 8 (CA cell bodies of the formatio reticularis of the mesencephalon, see Dahlstrom and Fuxe 1964)

5 HT pathways

By means of treatment with MAO inhibitors it has been possible to establish the existence of ascending 5 HT fibres to the telencephalon and diencephalon (Dahlström and Fuxe 1964) These fibres emanate mainly from 5 HT nerve cells of the nuc raphe dorsalis and nuc raphe medianus of the mesencephalon (B 7 and B 8 according to Dahlstrom and Fuxe 1964) and probably also from 5 HT nerve cells of the formatio reticularis of the mesencephalon (B 9 according to Dahlstrom and Fuxe 1964) Most of these fibres enter the medial forebrain bundle Final evidence for the existence of such ascending 5 HT neurons was obtained by making lesions of this bundle (Anden *et al* 1965 b) At least 75 per cent of the 5 HT nerve terminals in the diencephalon and telencephalon arise from 5 HT fibres ascending in the area of the medial forebrain bundle since this amount of 5 HT was lost at most on the operated side but not on the unoperated side (Table III) Marked decreases in number of

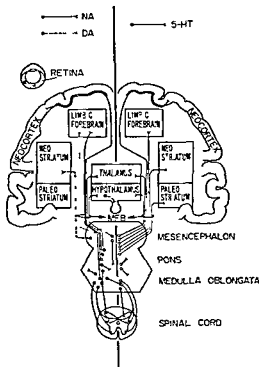


Fig 10 Schematic drawing showing, in highly simplified form, the main monoamine neuron systems in the central nervous system

5-HT terminals were observed *e g* in the globus pallidus, the septal area, the amygdaloid complex, the hypothalamus, and the gyrus cinguli Recent results of the effect of lesions of the 5 HT cell bodies in group B 7 and B 8 show that at least most of them give rise to ascending 5-HT fibres The 5-HT axons to the neocortex were observed after removal of this structure and they take a course similar to the corresponding NA axons *i e* they pass from the medial forebrain bundle in a dorsal direction in front of the septal area, partly via the tractus diagonalis, into the superficial part of the white matter of the neocortex

Discussion

The demonstration and mapping out of the monoamine neurons ascending from the lower brain stem to the telencephalon and diencephalon are largely based on anterograde and retrograde changes in the neurons following lesions in different parts of them The evidence of these observations will be briefly discussed below

After removal of the DA terminals in the neostriatum the non terminal DA axons can be traced from the area of the DA cell bodies in the substantia nigra to the region containing the terminals Ordinarily 24 hrs after reserpine treatment the monoamine nerve cell bodies are known to show fairly strong fluorescence while the terminals remain non fluorescent Not even under these conditions, however, can any DA nerve cell bodies be observed in the neostriatum Therefore the disappeara

of the DA terminals and DA of the neostriatum after destruction of the DA cell bodies in the substantia nigra or destruction of the DA axons in the rostral crus cerebri and in the internal capsule, should be due to anterograde changes in nigro neostriatal DA neurons The initial swelling and the subsequent atrophy and chromatolysis observed in the DA nerve cells of the substantia nigra after damage to the ascending DA axons must be interpreted as retrograde changes in these neurons The increased fluorescence intensity seen shortly after axotomy in the DA cell bodies of the sub stantia nigra and in the DA axons proximal to the lesion is probably a consequence of a piling up of amine storage granules formed in the cell bodies (Dahlstrom Fuxe and Hillarp 1965) When the cell bodies eventually degenerate after axotomy the capacity to form amine storage granules and amine synthesizing enzymes is reduced causing a decrease in their amine levels

After MAO inhibition the entire 5 HT neuron can be visualized under the fluorescence microscope (Dahlstrom and Fuxe 1964 1965) By this method it has been observed that most of the 5 HT cell bodies in the mesencephalon give rise to axons ascending in the medial forebrain bundle Sometimes even the way in which the 5 HT non terminal axons are transformed into 5 HT nerve terminals can be seen directly Therefore the loss of the 5 HT terminals and 5 HT of these regions after section of the medial forebrain bundle should represent anterograde degenera tive changes in the 5 HT neurons damaged by the lesion In addition there is an accumulation of 5 HT in swollen nerve fibres immediately proximal to the lesion similar to the accumulation observed in the nigro neostriatal DA neurons

After removal of large parts of the neocortex the non terminal NA and 5 HT axons innervating the neocortex are visualized and can be traced to the medial forebrain bundle Practically no monoamine nerve cell bodies have been detected in or rostral to the medial forebrain bundle not even after treatment with MAO inhibitors and/or the monoamine precursors L 3 4 d hydroxyphenylalanine or DL 5 hydroxy tryptophan From these data it is evident that the losses of the NA and 5 HT nerve terminals as well as NA and 5 HT of the various regions of the telencephalon and diencephalon after medial forebrain bundle lesions are due to anterograde degene rative changes in monoamine neurons

Independent studies by Heller and coworkers (Heller Harvey and Moore 1962 Heller and Moore 1965) have also shown that in rat brain there is a loss of 5 HT and NA after medial forebrain bundle lesions They have also found in the cat a dis appearance of 5 HT and NA in the neocortex (Moore Wong and Heller 1965 Heller Seiden and Moore 1966) After destruction of the medial forebrain bundle these investigators did not find any degenerating nerve fibres to the neocortex using the Nauta silver staining technique It was therefore assumed that the decreases in amines of the neocortex were not caused by interruption of monoamine neurons leading to this structure Instead they suggested that the changes in *e g* the neocortex are caused by transsynaptic effects in monoamine neurons These neurons were thought to be in polysynaptic contact with fibres ascending in the medial forebrain bundle However in the present study it was shown by direct methods that there

are ascending monoamine fibres in the medial forebrain bundle to the neocortex and that the neocortex does not seem to contain any monoamine nerve cell bodies By means of the histochemical fluorescence method it has also been observed that the ascending fibres are very thin (1—2 μ) and seem to be unmyelinated These facts may explain why Heller *et al* have failed to detect fibre degeneration in the neocortex

It has been known for a long time that peripheral organs are depleted of NA after postganglionic sympathectomy (Euler and Purkhold 1951, Goodall 1951) After preganglionic sympathectomy, on the other hand, there is no definite reduction of the NA content (Rehn 1958) Thus the NA and 5 HT terminals as well as the amines in both the central and peripheral nervous system disappear after lesions of the neurons to which they belong The L 3,4 dihydroxyphenylalanine/5 hydroxytryptophan decarboxylase activity has also been found to be lost from peripheral organs after postganglionic but not after preganglionic sympathectomy (Anden, Magnusson and Rosengren 1965) Loss of this activity also occurs in the spinal cord caudal to a transection (Anden, Magnusson and Rosengren 1965), in the whole brain after medial forebrain bundle destruction (Heller *et al* 1965) and in the neostriatum after lesions of the nigro neostriatal DA axons (see Results) This shows that also an enzyme participating in the monoamine synthesis disappears after anterograde degeneration of monoamine neurons indicating that it is present in monoamine nerve terminals

The principles described in this paper have been employed in these laboratories for about three years to demonstrate and map out the central monoamine neurons The findings obtained are tentatively and schematically presented in Fig 10 The bulbospinal pathways are best known (Carlsson *et al* 1964, Andén *et al* 1964 b, Dahlstrom and Fuxe 1965) In the present paper the distribution of the ascending fibres from the lower brain stem have been elucidated By selective lesions of the individual monoamine nerve cell groups it will, in all likelihood, be possible to determine in detail to which cell bodies the monoamine nerve terminals of the various brain regions belong, in the same way as has been shown for the nigro neostriatal DA neurons

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Responses of Skeletal Musculature and its Vasculature During "Diving" in the Duck: Peculiarities of the Adrenergic Vasoconstrictor Innervation

By

B FOLKOW, K FUXE and R R SONNENSCHEIN[1]

Abstract

FOLKOW, B, K FUXE and R R SONNENSCHEIN *Responses of skeletal musculature and its vasculature during "diving" in the duck Peculiarities of the adrenergic vasoconstrictor innervation* Acta physiol scand 1966 67 327—342

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[illegible] produced considerable flow increases also during vasoconstriction further maximal flow reached only some 50 ml Simultaneous recordings of venous outflow and pressures in
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In duck muscles maximal contractile force increased during CO_2 breathing due to augmentation via the sympathetic innervation (Orbeli effect) an effect mimicked by intra arterial catechol amines The ability of diving animals to maintain muscle vasoconstriction during exercise while diving may well reside in the differently balanced vascular innervation with intense constrictions of extramuscular vessels The sympathetic augmentation of contractility may partly compensate for the effect of flow reduction The larger maximal flow capacity and capillary area in duck muscles facilitates rapid post-dive restitution

Adaptations of animals to altered environments are no more strikingly illustrated than by the cardiovascular adjustments that occur in diving animals during submersion A common feature is the rapid development of bradycardia presumably associated with a marked reduction in cardiac output and an intense vasoconstriction in most tissues other than the brain and the heart cf Scholander 1940, 1962), though there are some disagreements in the literature in this respect, as will be discussed later The adaptive value of this response pattern during diving is evidently

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the preservation of available oxygen for the most oxygen-dependent organs, the heart and the nervous system.

Some aspects of nervous adjustments involved in the "diving reflex", including the nature of the stimuli and receptors, have been worked out. In ducks, this reflex response, integrated at the brain stem level, may be initiated through immersion *of the head alone, being strongly reinforced by the progressive increase in carbon* dioxide and decrease in oxygen tension (cf. Folkow and Fengl 1965, Andersen 1963 a, b, c).

On the other hand, little is known of the particular adaptations which may reside in the peripheral vessels and their innervation. Particularly with regard to the relations between activity and blood flow in the muscles several interesting questions arise. What relations exist between the "metabolic" vasodilatation during work and the superimposed nervous vasoconstrictor influence? Certainly diving animals often perform considerable exercise while submerged. If they were to behave in this respect as do man and cat, the metabolic vasodilatation should overcome sympathetically induced constriction (Blair, Glover and Roddie 1961, Kjellmer 1965) which would largely nullify the "protective" value of the vasoconstriction during diving. Therefore it is most likely that in diving species the metabolic effect does not "break through" the reflex constriction. What then is the basis for the difference between diving and non-diving animals? Furthermore, what effects do a severe restriction of blood flow have on the capacity of the skeletal muscles to perform their work and in what ways may the muscles compensate for such a flow restriction?

It was towards an elucidation of these and related questions that this investigation was directed. The domestic duck was used both because it is easily obtained and because considerable information is available on characteristics of its diving response (Scholander 1940, Folkow and Fengl 1965, Andersen 1963 a, b, c). For comparative purposes, domestic turkeys were used as examples of non-diving birds, and cats, including results already published (Hirvonen and Sonnenschein 1962, Kjellmer 1965), as examples of non-diving mammals. — Some preliminary results have been presented (Sonnenschein and Folkow 1965).

Methods

Ducks 2.5—3.0 kg and turkeys 4—6 kg were anaesthetized as [illegible] cats [illegible]—3.0 kg [illegible] with pentobarbital sodium [illegible] per kg [illegible] additional amounts were given at intervals as necessary.

In ducks and turkeys (Fig. 1) the trachea was cannulated and the two vagal nerves, the two carotid arteries and the right jugular vein were isolated. The femoral artery and vein and the nerve supply to the calf muscles and the [illegible] were isolated in one leg, great care being taken not to damage the [illegible] The [illegible] muscles were tied off towards the [illegible] Thus [illegible] the femoral vessels [illegible] a few experiments, where the [illegible] maximal blood [illegible] were [illegible] nerve supply was very thoroughly [illegible] The femur was then [illegible] just above the knee [illegible] and its marrow [illegible] with bone wax. Then the [illegible] muscles were enclosed in a plethysmograph using the skin as a [illegible] seal, a principle [illegible] details identical with that used by Kjellmer 1965 for the cat's calf muscles.

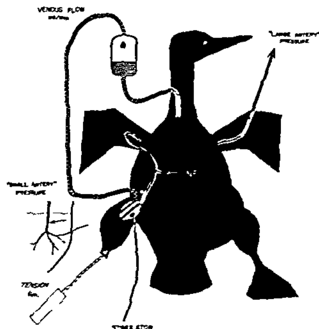


Fig 1 Diagram illustrating the experimental setup used in ducks Constant features were the recording of venous flow from the lower leg muscles and of arterial pressure from the brachial artery, and placement of electrodes around the intact sciatic nerve for stimulation. The inset illustrates the placement of a cannula in a branch (0.3—0.5 mm diam) of an artery, lying on the surface of a leg muscle for recording of "small artery" pressure.

[illegible] method In those experiments where CFC measurements were to be performed, or where [illegible] intra-arterial administration of drugs or control of arterial inflow was desired, flow to the femoral artery was directed by a cannula from the carotid artery Central arterial pressure was recorded via a polyethylene catheter usually inserted into the brachial artery with its tip placed in the aorta. In most cases a mercury manometer was used, writing on a kymograph

In one group of experiments the pressure gradient along the "large" arteries was determined in ducks, cats and turkeys by a technique similar to that described by Haddy *et al* (1954) Simul- [illegible] purposes of calculation of resistance flow through the arterial section between the two points of measurement was considered to be proportional to changes in total calf blood flow Resistance in the segment between the "central arterial pressure and "small artery pressure" is referred to as "large artery resistance"

Muscle work was induced by stimulation of the sciatic nerve, with isolated bipolar silver electrodes enclosing the nerve Supramaximal stimuli 1 to 4 per second, were such as to produce either single twitches (single square wave impulses of 0.5 to 1.0 msec or brief tetani 20—40 msec trains of 0.02 msec pulses with spacing of 5 msec between pulses When muscle force was to be recorded the tendons of the flexor muscles were cut and a stiff wire was tied firmly into the tendons of the extensor muscles This wire was connected to a Grass FT 10 tension transducer recording on a Grass Polygraph Both the transducer and the animal's tibia or knee joint were clamped firmly to minimize movement and allow near isometric recording of tension

[illegible] could not be utilized for [illegible] in both ducks and [illegible] desired, the reflex [illegible] al vagotomy and temporary bleeding as the arterial pressure was rising due to the vagotomy it was technically impossible unfortunately to expose the sympathetic chain for direct graded stimulations in

the birds but by suitably balancing the above-mentioned procedures fairly stable intense if desired reflex vasoconstrictions could be induced

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at 2—16 per sec)

The adrenergic nerve terminals in the femoral artery and vein in their branches and tributaries outside and within the lower limb muscles were studied with the help of the histochemical fluorescence method for the demonstration of catechol amines and 5 hydroxytryptamine (Falck *et al* 1962) Pieces from the above-mentioned areas were dissected out from 3 ducks and 3 turkeys freeze dried treated with formaldehyde gas embedded sectioned longitudinally and transversely and studied in comparison with similar previously described preparations from cats (Fuxe and Sedvall 1965) A large number of sections of the different specimens were stained with Azan for general histologic study

Results

The following observations resulted from a total of 40 successful experiments on ducks, turkeys and cats

A *Vascular Response Patterns*

1 *General characteristics of blood flow in muscles of the duck at rest and during exercise* Measurements of blood flow and CFC during rest and at maximal dilatation as well as histological examinations revealed a greater vascularity in the duck than in the other species The resting flow in the duck muscle was commonly 30—50 ml/min/100 gm tissue (Fig 2 and 3), more than three to five times as great as in the turkey or cat, and the venous blood was bright red Only slightly higher values were seen in acutely denervated duck s muscle, suggesting that the resting' sympathetic discharge to the muscle vessels was generally low or even insignificant under the present experimental circumstances Resting CFC values in the duck were around 0 05 as compared with 0 010—0 015 in resting cat s muscle

Upon stimulation of the muscles of the duck a prompt hyperemia ensued, reaching maximal levels of 140—150 ml/min/100 gm tissue at a pressure head around 100 mm Hg (Fig 2) This, again, was about 3 times the maximal flow occurring in cats (compare Fig 2 and 4) and turkeys Also CFC was now around 0 15 in the duck as compared with 0 04—0 05 in the cat at maximal vasodilatation After the stimulation was stopped flow returned to the resting level in 1—3 minutes

2 *Reflex vasoconstriction* Administration of 20 % CO_2 resulted in the ducks in a gradually rising arterial pressure and a resistance increase of 200—300 per cent in the muscles, reaching its maximum in 3 to 5 minutes Bilateral vagotomy greatly augmented this vasoconstrictor effect and arterial pressure rose markedly Graded bleeding until arterial pressure had returned towards 100 mm Hg (withdrawal of up to 20—25 ml of blood per kg b w) further intensified this reflex vasoconstriction with muscle flow reduced to 1 ml/min/100 gm or less i e to less than 1/150 of the maximal blood flow capacity (see end of right panel of Fig 3) Upon cessation of CO_2 administration the vasoconstriction subsided, the arterial pressure dropped precipitously and the duck might have died unless the withdrawn blood were

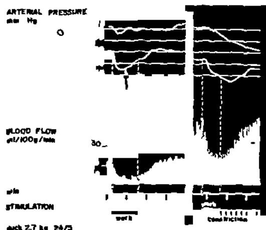


Fig 2 Central arterial pressure (top) "small artery pressure (center) and blood flow (bottom) in the lower leg muscles of a duck *Left panel* Pressure gradient of 40 mm Hg along the large artery during rest marked increase in the gradient at onset of work hyperemia, indicating intra muscular dilatation with subsequent reduction in the gradient (arrow) indicating ascending dilatation *Right panel* Moderate constriction during CO administration during superimposed work with moderate hyperemia the pressure gradient increased as before, but now with no tendency towards a secondary reduction i e no ascending dilatation

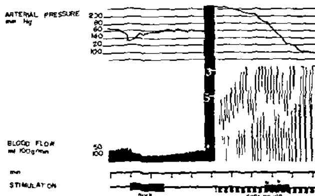


Fig 3 Arterial pressure and blood flow in the lower leg muscles of a duck *Left panel* Resting blood flow and increase during exercise *Right panel* Marked flow reduction during administration of 20 % CO_2 and graded bleeding ("constriction") only slight increase of flow during superimposed work Note that scales for blood flow are different in the two panels and that flow at right end of the right panel is far below 3 ml/100 g min

reinfused Attempts to produce similar vasoconstriction in cats or turkeys by administration of 20 % CO_2 were unsuccessful

3 *Interactions between exercise vasodilatation and sympathetic vasoconstriction* In the presence of maximal exercise hyperemia the superimposition of the strong reflex sympathetic discharge induced as described above under 2 resulted in a marked, usually well sustained reduction in flow in the duck sometimes nearly to the level obtained by the vasoconstrictor reflex alone If the reflex sympathetic discharge was less marked its interference with the maximal work hyperemia was relatively less pronounced

When the order of the procedures was reversed a maximal activation of the muscles could produce only a minimal increase in flow if the reflex vasoconstriction was intense Fig 3 right panel On the whole lesser degrees of vasoconstriction had correspondingly lesser restrictive effect on the development of exercise hyperemia in the duck but rarely was the metabolic dilator effect able to "break through" to any considerable extent beyond the resting blood flow level (Fig 2, right

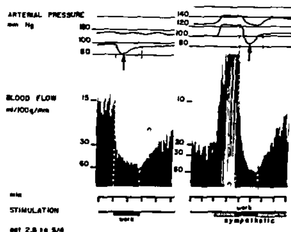


Fig 4 Results of an experiment on a cat comparable to that on a duck shown in Fig 2 *Left panel* Pressure gradient (20 mm Hg) and flow in the lower leg muscles during rest and superimposed work increase in the gradient at onset of work with subsequent reduction in the gradient indicating start of ascending dilatation (arrow) *Right panel* Constriction produced by stimulation of sympathetic chain at six impulses per second Note reduction in pressure gradient, indicating that constriction mainly involved intra muscular vessels with superimposed work increase in the gradient occurred with subsequent decrease, indicating start of ascending dilatation (arrow) as in the absence of sympathetic constriction

Our findings on cats confirmed those of Kjellmer (1965), indicating that vasodilatation produced by exercise can here overcome to a very large extent even intense neurogenic constrictions (Fig 4) In this regard, a large quantitative difference was found to exist between ducks and cats

4 *Pressure gradients in the "large" extramuscular arteries* The gradient between the "central" pressure and that in the 0 3—0 5 mm extramuscular artery was measured in 9 ducks, 5 cats and 2 turkeys during rest, muscle work, vasoconstriction and combinations of these procedures, in all cases total blood flow in the calf muscles was recorded simultaneously

During rest in ducks the resistance along the large arterial segment was on an average 30 % of the total resistance (range 8 to 60 %, above 20 % in 6 of 9 animals) On the other hand, in cats it was 12 % of the total (range 4 to 23 %) while in the two turkeys it was 12 % and 22 %, respectively

Upon vasoconstriction the *proportion* of resistance residing in the large arteries decreased somewhat in the ducks, but not nearly as much as in the cats (see Table I, where for comparative purposes tenfold increases of total resistance were selected for both species) However, since the neurogenic increase in total flow resistance was generally far greater in the ducks than in the cats, where it only exceptionally reached a tenfold increase, the absolute increase in large artery resistance was considerably more pronounced in the former species For instance, in one duck, where total resistance increased more than 20 fold, large artery resistance which here constituted approximately 25 % of the total, rose from 0 4 PRU to 8 PRU, a 20 fold increase Still larger increases of big artery resistance presumably occurred in those ducks where total resistance rose as much as 30 fold

Another difference was apparent between ducks on the one hand, and cats and turkeys, on the other, during dilatation produced by muscular work, under circumstances where no or only slight vasoconstrictor fibre activity was present (Table I) While the proportion of large artery resistance increased to only a small extent in

TABLE I Resistance in "large" and "small" arteries supplying the skeletal muscles in ducks and cats during rest, vasoconstriction and moderate exercise For comparative purposes 10-fold increases in total resistance are selected, which in ducks represents only a moderate vasoconstriction but in cats a very intense one Likewise, in moderate exercise reductions to 50 per cent of the resting flow resistance were chosen, an extent of vasodilatation seen in the duck only in situations with no ("ascending dilatation") or low sympathetic activity ("no ascending dilatation") Concerning the situation at intense sympathetic activity see Fig 7, right panel — The values are derived from 9 ducks and 5 cats, in each case total resistance during constriction and exercise was extrapolated or interpolated to the selected range and other values adjusted accordingly

Independent of sympathetic activity ascending dilatation always occurred during exercise *in cats, while in ducks it was seen only when sympathetic activity seemed to be insignificant* — Note particularly the greater increase in 'large artery' resistance during vasoconstriction in ducks

			3 Moderate exercise (flow doubled)	
	1 Rest	2 Vasoconstriction	Ascending dilatation	No ascending dilatation
Duck				
a. Flow (ml/100 g/min)	40	4	80	80
b Total resistance (R) [mmHg/(ml × 100 g/min) PRU]	2 5	25 (×10)	1 25 (×0 5)	1 25 (×0 5)
c Proportion of R lying in the 'large artery"	30%	20%	35%	55%
d R in "large artery", PRU	0 75	5 0 (×7)	0 45 (×0 6)	0 7 (×0 9)
e R in "small vessels', PRU	1 75	20 (×12)	0 8 (×0 45)	0 55 (×0 3)
Cat				
a Flow	10	1	20	
b Total R, PRU	10	100 (×10)	5 (×0 5)	
c Proportion of R in "large artery'	12%	4 5%	17%	
d R in "large artery", PRU	1 2	4 5 (×3 5)	0 9 (×0 75)	
e R in 'small vessels", PRU	8 8	95 5 (×11)	4 1 (×0 45)	

this situation in all cats and the two turkeys (from average of 12 % up to 17 %), it was initially higher and increased often more than two times as much in the ducks (from average of about 30 % up to about 55 %) That is to say, for a given reduction in total resistance during work, the amount of ascending dilatation in the large arteries of ducks was considerably less than in cats and turkeys Fig 2 (left panel) illustrates the occurrence of such an ascending dilatation (arrow) in the large artery of a duck, as judged from the delayed decrease in large artery resistance when muscle work was started

If vasoconstrictor fibre activity was superimposed the difference between ducks and cats became even more marked The right hand panel of Fig 2 illustrates how even a moderate reflex vasoconstriction in the duck abolishes the ascending

dilatation, as judged from the fact that now the increase in pressure gradient between "central" and "small artery" pressures remained throughout the exercise hyperemia Frequently the proportion of large artery resistance in the duck was markedly increased during exercise if an intense reflex sympathetic activity was present as is illustrated in Fig 3 and 7 Fig 4, on the other hand, shows how in the cat an essentially unchanged ascending dilatation (arrow) occurs during muscle work, whether the vasoconstrictor fibres were inactive (left panel) or strongly stimulated (right panel)

B *Distribution of Adrenergic Nerve Terminals to the Blood Vessels Supplying the Calf Muscles*

In order to elucidate further the physiological results, it was found necessary to study the adrenergic innervation of the femoral artery and vein of the lower limb muscles of the duck and the turkey and compare them with earlier observations on cats (Fuxe and Sedvall 1965)

1 *Femoral artery* The adrenergic ground plexus, built up of strands of adrenergic nerve terminals and surrounding but never penetrating the media, was found to be very dense in the duck, much more so than in the turkey where only scattered adrenergic terminals could be found, as in the cat (Fig 5) An interesting finding in the duck was that at some of the places, where branches left the femoral artery, there occurred invaginations of the entire wall into the lumen of the side branch The media of the invaginations was surrounded by a fairly dense plexus of adrenergic nerve terminals It may be that these invaginations, when under high sympathetic stimulation, are able to reduce drastically the blood flow to the sidebranch in question On the other hand, the sidebranches *per se* did not exhibit denser adrenergic innervation than the corresponding vascular sections in turkeys and cats

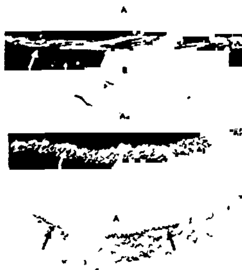


Fig 5 I (Upper) Femoral artery of duck Somewhat obliquely cut section A dense plexus of intensely fluorescent adrenergic nerve terminals (→) is present on the border between the media (A) and the adventitia (B) No adrenergic nerve terminals penetrate the media × 170
II (Middle) Femoral artery of turkey Cross section Relatively few cross sectioned bundles of adrenergic nerve terminals (→) are present surrounding the media (A) of the artery They appear as clusters of fluorescent dots which are somewhat difficult to observe due to a high background fluorescence of the collagen fibres × 170
III (Lower) Femoral artery of cat Cross section A few cross-sectioned bundles of adrenergic nerve terminals appearing as fluorescent dots (→) are present close to the media (A) × 170

Further, the femoral artery of the duck showed a considerably higher amount of elastic material than those of the turkey and the cat, which were closely similar

2 *Femoral vein* The femoral vein of the duck showed a somewhat richer innervation than that of the turkey, although the difference was not as pronounced as in the case of the femoral artery The strands of adrenergic nerve terminals lay in the adventitia close to the muscle cell layer

3 *Intramuscular vessels* There were no signs of a direct adrenergic innervation of the skeletal muscle of the duck and turkey As in the cat (Fuxe and Sedvall 1965) the adrenergic nerve terminals were distributed mainly to the small intramuscular arteries both in duck and turkey, surrounding the media without entering it The adrenergic nerve terminals showed no sphincter like formations In each intra muscular vessel of the duck the adrenergic plexus showed about the same density as in turkey or cat, but the duck muscles showed a definitely greater density and number of intramuscular arteries and veins It follows that the number of adrenergic nerve terminals per unit muscle mass is probably larger in the duck, implying in turn that more adrenergic transmitter is released at a given discharge rate This factor may be of relevance for the results described under C

In all the species only a few bundles of adrenergic terminals were found along the intramuscular veins and some of the larger veins showed no innervation at all Single bundles of adrenergic nerve terminals were found along the smallest pre and postcapillary vessels

C *Performance of Skeletal Muscles*

Any large reduction in blood flow, whether caused by a mechanical flow obstruction or by vasoconstriction, induced a reduction in the maximum tension that the muscles could produce during intermittent stimulation at frequencies of 1 to 4 per second For example, during a marked reduction in flow, such as is illustrated in Fig 3, right panel, the muscles were greatly weakened Grossly, this flow dependence of muscle work in ducks was similar to that observed in cats in these and previously reported experiments (Hirvonen and Sonnenschein 1962 Wright and Sonnenschein 1965)

However, at the beginning of CO_2 administration in the ducks, when little or no reduction in muscle blood flow had yet occurred since blood pressure often rose in proportion to the increased sympathetic discharge a rather marked augmentation of maximal muscle force ensued (Fig 6 A) This started within a few seconds of the beginning of CO_2 administration and in about 30 seconds reached its maximum of 25—40 % above the control level of force Following substitution of 20 % CO_2 with air, the muscle force returned to its previous level within 2 minutes These results were obtained in 8 of 13 ducks (3 of 5 failures occurred when a pump was used to maintain constant arterial flow which often led to thrombocyte aggregation, plugging of the vessels and deterioration of peripheral responses)

If, during the period in which the muscle force was augmented, the sciatic nerve was temporarily blocked by cooling, centrally to the electrodes, muscle f

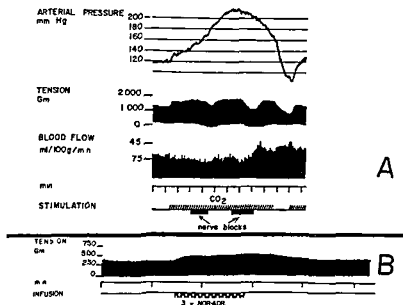


Fig 6 A Arterial pressure isometric tension of extensor muscles of lower leg during maximal 2 per sec stimulation of the intact sciatic nerve and muscle blood flow in a duck During the times indicated at the bottom CO_2 was administered and sciatic nerve blocks central to the stimulating electrodes were produced by temporary cooling the nerve Upon CO_2 administration muscle force increased to decrease again when CO_2 was stopped Each nerve block during CO_2 administration reduced muscle force to control level When the nerve was rewarmed muscle force rose again even if blood flow then sometimes decreased due to the returning sympathetic vasoconstriction B Augmentation of muscle force stimulated as above in a duck by intra arterial infusion of noradrenaline while muscle blood flow was held constant by adjustment of perfusion pressure

decreased to control level only to return again to the higher level when the nerve was rewarmed (Fig 6 A) This indicated, of course, that the effect was being mediated over the nerve rather than by a blood borne substance In two experiments CO_2 was given and the nerve was then centrally blocked by cooling When the new lower level of muscle force was attained, arterial flow was completely stopped by clamping the artery, whereupon muscle force rapidly decreased to an imperceptible level Then, while flow was still stopped, the nerve was rewarmed and contractions reappeared for 10—15 seconds, only to disappear again as a result of the flow obstruction Since there was no flow during the course of these observations the augmentation of contractility could hardly have been due to any altered flow pattern within the muscle

The possibility naturally suggested itself that the augmented muscle contractility was produced by the adrenergic transmitter, diffusing to reach the muscle fibres during the reflex vasoconstrictor fibre activation by CO_2 inhalation This was tested by intra arterial infusion of noradrenaline and adrenaline to ducks while blood flow to the calf muscles was held as nearly constant as possible Figure 6 B illustrates one such experiment in which noradrenaline induced an increase of up

to 40—50 % in contractility when flow was constant or slightly decreased Adrenaline produced similar effects For both agents the lowest rate of infusion (1 microg per min with blood flow at 22 ml per min) had no perceptible vascular effect but was slightly above threshold for increasing muscle contractility, 3 microg per min produced maximal effect on contractility

Discussion

These observations have thrown light on some of the physiological and anatomical adaptations of the peripheral vasculature and its sympathetic innervation, as well as of interaction between these and the performance of skeletal muscle, in the "diving" response of the duck Even under the present artificial conditions it is evident that a reflex increase in sympathetic discharge can produce a very intense vasoconstriction in the skeletal muscles of the duck, and that the metabolic dilatation accompanying muscle work can only insignificantly interfere with the constriction, once this is intense In "non diving" animals such as cat and man, on the other hand, the metabolic dilatation breaks through a neurogenic vasoconstriction to a far greater extent

The ability of the peripheral vasoconstriction to withstand the metabolic dilator influence in the duck appears to reside primarily in the characteristics of the larger extramuscular arteries These, in the first place, are relatively more narrow than corresponding vessels in the cat, this fact, along with the greater number of intramuscular small vessels, means that the extramuscular arteries in the duck contribute a much greater proportion of the total resistance than in the cat The evidence for this is the much larger pressure gradient between aorta and cannulated small artery in almost all ducks compared to cats (It must be emphasized that the cannulated "small arteries" were of the same size (0 3—0 5 mm) in both species) Coupled with this geometric property is the markedly more dense adrenergic innervation of the large arteries in the duck than in the cat (and turkey) and the far more powerful and well maintained constrictions of these vessels during sympathetic discharge, which in the duck seems to suppress entirely the ascending dilatation

With these characteristics in mind, the course of events during muscular work both without and with superimposed vasoconstrictor activity can be envisaged The accompying diagram (Fig 7 is an attempt to illustrate in an admittedly simplified and distorted way the quantitative differences among the events as they occur in the duck and the cat The situation at rest with low or insignificant sympathetic discharge, is much as described above with a greater pressure gradient along the large artery of the duck and with a simultaneous resting blood flow that is 3—5 times higher than in the cat thanks to the greater number of small intramuscular vessels The turkey resembles much more closely the cat than it does the duck, both in terms of the resting flow level and the density of small intramuscular arteries

During work without constrictor fibre activity locally produced vasodilator substances act directly on the small intramuscular vessels the ascending dilatation involving the larger extramuscular arteries is propagated from the periphery in-

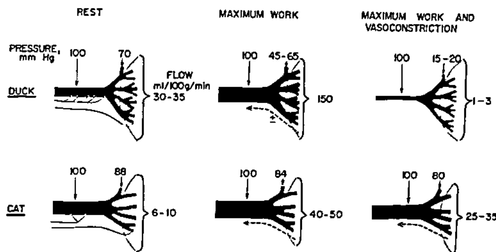


Fig 7 Diagrams illustrating the differences in innervation and geometry of extra- and intramuscular arteries of the duck and cat, the characteristic pressure gradients along the large, extramuscular arteries and the rates of blood flow in rest, maximal work and maximal work plus intense vasoconstriction The large artery of the duck is much more densely innervated, further there are more small arteries per unit muscle (indicated by the dotted line) than in the cat Values for the "small artery" pressures and for blood flows are approximate for the different situations The arrow indicates ascending dilatation, in the case of the duck during maximum work, ± means that the ascending dilatation is not a constant feature

both species However, the maximal blood flow capacity and total capillary surface area open for exchange in the duck seem to exceed those of cats and several other species by a factor of about three

When intense vasoconstriction is superimposed on the work, the differences between duck and cat become most apparent Although the small intramuscular vessels remain dilated to a considerable extent under the influence of the local metabolites, these metabolites do not reach the larger extramuscular arteries directly and can therefore not compete locally with the influence of their constrictor fibres During exercise these large arteries can only be widened by means of the indirectly mediated ascending dilatation which, however, is strongly suppressed in the duck but not so in the cat, probably a result of the more potent large artery innervation in the former species The resistance in these large arteries can thus be kept markedly increased and here the invaginations of some of the large arterial branches of ducks may greatly contribute This intense constriction of also the large arteries in ducks is in agreement with earlier observations of the constriction of arterial branches in the flippers of seals (Irving, Scholander and Grinnell 1941) Even though the neurogenically constricted small blood vessels dilate somewhat during excercise in the duck, the total resistance can nevertheless be kept far above the resting level by way of the 'throttle mechanism' inherent in the constriction of the large arteries In marked contrast to this situation the absence of such a throttle in the cat, i e the occurrence in this species of ascending dilatation and a bigger "break through" in the periphery, allows the total resistance to fall and the flow to increase markedly

during work, even in the presence of high sympathetic activity In all these respects the turkey is much like the cat

In other words, the quantitative difference in vascular arrangement and innervation so influence the competitive balance between neurogenic constriction and metabolic dilatation that in the duck the effectively operating constrictor fibre influence tends to strangle muscle exercise, rather than allow the exercise metabolites to overcome the neurogenic constriction, while the reverse tendency seems to hold for the cat (and turkey) While it is tempting to infer from these facts that the anatomical and physiological peculiarities of the vasculature of the duck are specific to its diving habit, cautious reserve must be maintained until other species, both diving and non diving, are investigated In any case, the central nervous regulation of vascular resistance in skeletal muscles appears to play a much more prominent role in ducks than in other species so far studied

It is further worth nothing that the important sympathetic supply to the large artery in the duck apparently courses for a considerable extent along the vessel wall, while the supply to the intramuscular vessels is contained in the peripheral nerve, at least up to the point where it enters the muscle This follows from the observation in several animals that section of the sciatic nerve failed to relieve neurogenic constriction occurring in the large arterial segment In the study of Hollenberg and Uvnas (1963), often no increase in muscle flow resistance occurred in the ducks during "diving" while most other investigators find evidence of a muscle vasoconstriction in ducks and other animals (Irving 1938, Scholander 1940, Grinnell, Irving and Scholander 1942, Andersen 1959, Johansen and Krog 1959) However, in the experiments of Hollenberg and Uvnas the insertion of the flow recorder in the artery must have interrupted the nerve fibres along this vessel, and these fibres are probably responsible for the intense and perhaps crucial constriction of the bigger arteries Further, the low 'resting' flow reported by these authors for muscle blood flow suggests that the sympathetic activity to the 'distal' part of the resistance vessels must have been very high at all times in their animals even when not 'diving' The neurogenic constriction of the bigger arteries is possibly the crucial additional factor during the induction of the diving reflex' if for other reasons, sympathetic activity to the small vessels is already high to start with The exceedingly low value for cardiac output sometimes seen while ducks are diving, and the immense post dive increase, observed in recent experiments (Folkow Nilsson and Yonce 1966), simply necessitates that muscle blood flow must be decreased to at most a few ml/100 g per minute, to reach maximal flow figures within 10—15 sec after cessation of the dive

One of the outstanding feature of the circulation in the muscles of the duck is the very high level of flow and large capillary surface area both at rest and during exercise as long as sympathetic activity is low and negligible at least 3 to 5 times the corresponding values in the cat and turkey and in man To judge from the visual observations (which must of course be quantified of the high saturation of venous blood during rest and most levels of work except the very maximal ones, this is indeed a "luxuriant flow whose significance is an intriguing problem By th

ful constrictor fibre control it can, however, effectively be set at almost any lower level depending on the extent of the sympathetic discharge, even to the extent of nearly stopping the flow entirely It is likely that this high capacity of flow rate must be, among other things, of importance in the post diving period to meet the need of a fast wash out of accumulated metabolitics (Scholander 1940, 1962) One might also speculate that in case "resting" blood flow is kept high just before a dive by a temporarily reduced constrictor fibre activity in this phase, the high oxygen content of the venous blood, a result of the high flow and correspondingly lowered extraction ratio, would create a larger dynamic venous reservoir of oxygen, available to the animal during the period of diving

The increase in contractility of the lower leg muscles of the duck during the reflex response to CO_2 is one of the more striking "adaptive" mechanisms encountered in this animal The evidence points strongly towards mediation of the effect over the sciatic nerve since temporary block of the nerve, centrally to the site of stimulation, either prevented or reversed the increase in developed tension induced by CO_2 breathing, and did so even if the blood flow was completely obstructed before hand It can be invoked that the sciatic stimulation might have activated also muscle spindle afferents, producing a reflex reinforcement of the muscle activation CO_2 might then by an action on the spinal reflex centers facilitate this superimposed reflex muscle activation so as to produce an increased muscle response during CO_2 breathing However, this cannot be the case as the induced muscle activation before CO_2 breathing was supramaximal in relation to the blood flow and therefore definitely flow-limited In such a situation a further intensification of the motor fibre discharge can only enhance muscle force for a few seconds, meaning that the point of action must be a peripheral one, occurring in the muscle itself

With respect to such a peripheral mechanism, one possibility is that some redistribution of blood flow within the muscle may occur, such that more effective conditions for transcapillary exchange would obtain and allow a higher level of performance by the muscle However, the occurrence of the augmenting effect even in the total absence of blood flow speaks against this mechanism as being of prime importance, although it cannot be ruled out as a contributory factor

It appears most likely that the augmentation is an example of the Orbeli effect, i e increase of contractility induced by sympathetic discharge (cf Tiegs 1953) It cannot be explained by a direct adrenergic innervation of the skeletal muscle since, as shown in the present study, the adrenergic nerve terminals are distributed exclusively to the blood vessels Rather, it is necessary to postulate that the sympathetic amines are able to diffuse out from the adrenergic nerve terminals and to reach the skeletal muscle fibres in concentrations high enough to affect the fibres, that these amines can affect contractility of skeletal muscles has been previously demonstrated (cf Bowman and Zaimis 1958) It is known from experiments in cats that even though during muscle rest the adrenergic transmitter is locally eliminated, mainly by means of active reuptake into the terminals, during exercise it diffuses out in considerable amounts into the tissue spaces and the blood stream

(Carlsson, Folkow and Haggendal 1964) In the cat, however, the total amount released during physiological discharge rates is apparently too small to affect muscle contractility significantly, as in this species a clearly evident Orbeli effect is not seen under these circumstances Bulbring and Burn (1939) demonstrated the Orbeli effect in muscles of the dog, but only with stimulation of the sympathetic nerves at markedly supraphysiological frequencies when an excess release of transmitter occurs

In the duck, however, the vascular bed within the skeletal muscle is much more dense, while the degree of innervation of the individual vessels is at least as intense as in the cat It follows that the total amount of adrenergic transmitter released at a given discharge rate per unit muscle weight and, hence, the local concentration of diffusable transmitter available around the skeletal muscle fibres of the duck may be expected to be decidedly higher than in the cat In addition it is, of course, also possible that duck muscles react more strongly to a given amount of catechol amines, which the catechol infusion experiments might indicate In any case, this local reinforcement of muscle contraction, evidently mediated by the locally released constrictor fibre transmitter, may help maintain performance of the skeletal muscles at a reasonable level, despite a severe reduction in their nutritional blood supply This may represent, then, a situation in which the Orbeli effect plays a physiological role, the first such situation of which we are aware

While no doubt many other adaptations to the diving habit have developed in ducks and other diving animals, these specializations in structure and function of the peripheral blood vessels and their innervation, and in the potentiality for increasing the work output of skeletal muscles, would appear to be of great importance

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Release of Free Fatty Acids from Subcutaneous Adipose Tissue in Dogs Following Sympathetic Nerve Stimulation[1]

By

SUNE ROSELL

Abstract

ROSELL, S *Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation* Acta physiol scand 1966 *67* 343—351

A technique suitable for quantitative measurements of circulatory and metabolic processes in subcutaneous adipose tissue *in vivo* is described Electrical stimulation of sympathetic nerves to the adipose tissue induces a net release of FFA as well as vasoconstriction A maximal release rate of FFA seems to be obtained with a stimulus frequency of about 3/sec The vasoconstrictor reaction following sympathetic nerve stimulation supresses the release rate of FFA This is especially true following stimulation with higher frequencies of 5/sec, or more Provided the stimulation frequencies are within the physiological range the stimulation period must be 2 min, or more, in order to produce a rise in the release rate of FFA The data indicate that sympathetic nerves to subcutaneous adipose tissue have the ability to mobilize energy for the organism within a few minutes

Several investigators have offered suggestive evidence that the sympathetic nervous system, to some extent, regulates the fat metabolism in adipose tissue Thus, denervation impairs the mobilization of fat from brown and white adipose tissue (Wertheimer 1926, Beznák and Hasch 1937, Hausberger 1935) Systemic administration of ganglionic blocking agents depresses the plasma level of free fatty acids (FFA) in men and dogs (Bogdonoff *et al* 1959, Havel and Goldfien 1959) The experiments by Correll (1963) give evidence of a more direct nature that sympathetic nerves to adipose tissue may regulate the mobilization of FFA Correll showed that electrical stimulation of nerves to isolated rat and rabbit adipose tissue *in vitro* enhances the release of FFA This response was prevented by chronic sympathetic dernervation, indicating that the release of FFA is due to an activation of nerves of sympathetic origin Moreover, the plasma level of FFA rises when certain diencephalic and mesencephalic areas connected to the sympathetic nervous system are electrically

[1] A preliminary report on parts of the material presented here has been published elsewhere (Oro, L. S Rosell and L. Wallenberg Nature 1965 *205* 178)

stimulated in dogs (Oró, Wallenberg and Bolme 1966) Furthermore, the plasma level of FFA increases (Dole 1956, Gordon and Cherkes 1956, 1958) following the infusion of the sympathetic transmitter noradrenaline

The present experiments were carried out to study, *in vivo*, the quantitative relationship between the stimulation of sympathetic nerves and the net relaese of FFA from subcutaneous adipose tissue in dogs

Methods

Experiments were conducted on 20 [illegible]

during the perfusion After completion of the preparation the skin was replaced to maintain warmth and moistness

The adipose tissue was perfused via the artery at a constant flow rate with defibrinated blood from the same dog by means of a perfusion technique described earlier (Renkin 1961 Renkin and Rosell 1962) To defibrinate blood a femoral artery was cannulated and the blood was collected in a glass beaker while stirring with a whisk The reservoir of the perfusion apparatus was then filled with the defibrinated blood The blood loss of the dog was compensated with 0 9 % NaCl i v No heparin was administered The hematocrit of the perfusion blood was on the average 0 31 [illegible]

frozen for later analysis of FFA The blood pressure of the dog was recorded from the common carotid artery and the arterial perfusion pressure from a cannula connected to the perfusion apparatus with Statham pressure transducers (23 AA) The perfusion pressure was adjusted to approximately the same level as the arterial blood pressure of the dog The pressures and the rate of perfusion flow were recorded on a Grass Polygraph model 5B At the end of the experiment the adipose tissue was excised and weighed The tissue prepared as described had a mean weight of 49 g (range 22—100 g)

FFA was determined according to Dole (1956) as modified by Trout Estes and Friedberg (1960) FFA was determined in each venous sample and in two samples taken from the perfusion blood one sample at the beginning of the perfusion and another at the end The mean of the two samples was used as the arterial FFA concentration The net uptake or net release of FFA was calculated as the arteriovenous FFA difference times the plasma flow

In two experiments the ipsilateral sympathetic chain was exposed through a midline abdominal incision and transected above L_1 The peripheral end was stimulated at the level of L_1—L_2 In the rest of the experiments the nerve to the adipose tissue was cut at the level of the external hiatus of the inguinal canal and the peripheral end was stimulated The nerves were stimulated with square pulses from a Grass S4 stimulator through bipolar silver electrodes 2 mm apart Stimulation intensities and pulse durations supposed to be supramaximal were used (4 8 V and 10 msec respectively)

Results

I Resting values

At the beginning of the perfusion run the pressure was adjusted to about the level of the arterial blood pressure This resulted in a mean blood flow of 6 ml/min 100 g (range 2 1—13 5)

Prior to the perfusion run the nerve to the adipose tissue was transected at the external hiatus of the inguinal canal Venous blood samples were then collected

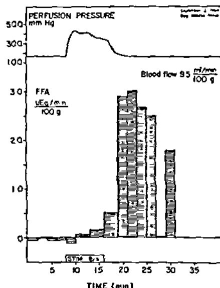


Fig 1 Dog 20 kg Nembutal anesthesia Adipose tissue 46 g Constant blood flow 9 5 ml/min 100 g Arterial FFA concentration 0 23 μeq/ml plasma Stimulation of sympathetic chain 6 V, 8/sec for 8 min Sample no 13 was destroyed Downward bars from baseline indicate net uptake and upward bars net release

prior to the stimulation of the peripheral end of the transected nerve The sample taken prior to the first stimulation period was chosen to determine the resting value of the net flux of FFA In 5 out of 29 expts there was a small net uptake of FFA, in 15 expts a net release prevailed, while in 9 experiments there was no significant net flux found The mean value of all the experiments showed a net release of 0 17 ± 0 05 μeq/min · 100 g (Mean ± SEM) The FFA concentration in the arterial perfusion blood was 0 28 ± 0 06 μeq/ml plasma (Mean ± SEM)

Comments to I

Since adipose tissue is capable of taking up FFA as well as of releasing it to the circulating blood, (Shapiro, Chowers and Rose 1958, Fredrickson and Gordon 1958, Raben and Hollenberg 1960) the data presented indicate the net value of the influx and outflux of FFA The adipose tissue was denervated in most of the experiments prior to the perfusion run and, therefore, deprived of nervous activity which is partly responsible for the outflux of FFA This would tend to result in a prevailing influx of FFA, and may be one of the reasons why a net uptake was seen in 5 of the experiments

II *Stimulation of the sympathetic trunk or the peripheral nerve to the adipose tissue*

In two experiments the sympathetic trunk was stimulated at the level of L_1—L_2 while the peripheral nerve to the adipose tissue was left intact Fig 1 is an illustration from one of these experiments The sympathetic chain was stimulated for 8 min with a frequency of 8/sec During the stimulation period the perfusion pressure increased as a result of vasoconstriction Following cessation of the stimulation there was a pronounced net outflow of FFA with a peak value of about 3 μeq/min 100 g The

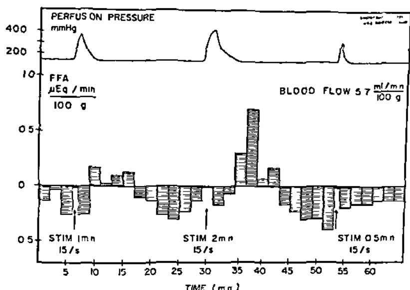
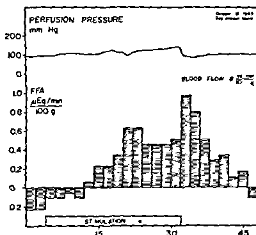


Fig 2 Dog 25 kg Nembutal anesthesia Adipose tissue 79 g Constant blood flow 5 7 ml/min 100 g Arterial FFA concentration 0 19 μeq/ml plasma Stimulation of the nerve to the adipose tissue 7 V 15/sec for 1 min 2 min and 0 5 min respectively

outflow decreased slowly and amounted to about 2 μeq/min 100 g 15 min after the cessation of the stimulation In the remainder of the experiments the nerve supplying the adipose tissue was stimulated and it was observed that the two types of stimulations produced qualitatively the same results

The effects of different stimulation periods and stimulation frequencies were studied systematically to determine the optimal stimulation parameters necessary to induce the release of FFA Fig 2 illustrates one experiment in which the stimulation period was varied A stimulation frequency considered to be supramaximal (15/sec) was chosen There was a net uptake of FFA prior to the stimulation Following nerve stimulation for 1 min, there was a small net release of FFA for 8 min, while stimulation for 2 min resulted in a clearcut net release However, no significant response was noticed if the stimulation period was 0 5 min Other experiments indicated that provided the stimulation frequency is less than 15/sec the stimulation period has to be 2 min or more, in order to produce a rise in the FFA release In one series of 12 dogs the nerve was stimulated for 24—30 min with a low constant frequency (1—10/sec) Stimulation with low frequencies may provide information about the magnitude of FFA release resulting from activity in the sympathetic nervous system under physiologic conditions As demonstrated in Fig 3 stimulation with 1/sec for 28 min resulted in a gradual rise in FFA outflow which reached a level of about 0 5 μeq/min 100 g Following the cessation of the stimulation the FFA outflow increased still further before returning towards the prestimulation level However, the major part (about 60 per cent) of the net release occurred during the stimulation period The perfusion pressure did not change appreciably during the



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for 28 min

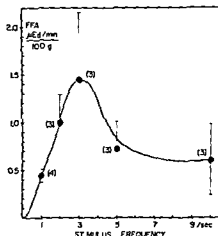


Fig 4 Relation between stimulation frequency and the rate of net release of FFA The net release rate is the value obtained *during* the stimulation period of 24 to 30 min. The figures within parentheses indicate the number of stimulations The vertical bars represent ± SEM

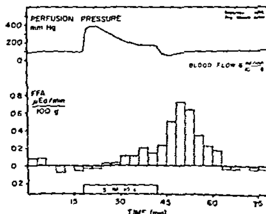


Fig 5 Dog 8 kg Nembutal anesthesia Adipose tissue 40 g Constant blood flow 6 ml min 100 g Arterial FFA concentration 0 28 μeq/ml plasma Stimulation of the nerve to the adipose tissue 8 V 10 sec for 24 min.

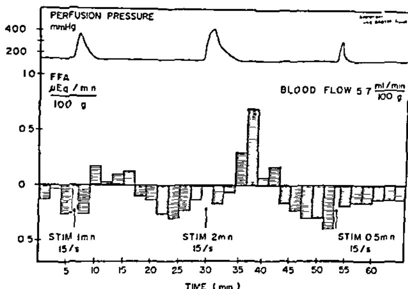


Fig 2 Dog 25 kg Nembutal anesthesia Adipose tissue 79 g Constant blood flow 5 7 ml/min 100 g Arterial FFA concentration 0 19 µeq/ml plasma Stimulation of the nerve to the adipose tissue 7 V 15/sec for 1 min 2 min and 0 5 min respectively

outflow decreased slowly and amounted to about 2 µeq/min 100 g 15 min after the *cessation of the stimulation In the remainder of the experiments the nerve supplying* the adipose tissue was stimulated and it was observed that the two types of stimulations produced qualitatively the same results

The effects of different stimulation periods and stimulation frequencies were studied systematically to determine the optimal stimulation parameters necessary to induce the release of FFA Fig 2 illustrates one experiment in which the stimulation period was varied A stimulation frequency considered to be supramaximal (15/sec) was chosen There was a net uptake of FFA prior to the stimulation Following nerve stimulation for 1 min, there was a small net release of FFA for 8 min, while stimulation for 2 min resulted in a clearcut net release However, no significant response was noticed if the stimulation period was 0 5 min Other experiments *indicated that provided the stimulation frequency is less than 15/sec the stimulation* period has to be 2 min or more, in order to produce a rise in the FFA release In one series of 12 dogs the nerve was stimulated for 24—30 min with a low constant frequency *(1—10/sec)* Stimulation with low frequencies may provide information about the magnitude of FFA release resulting from activity in the sympathetic nervous system under physiologic conditions As demonstrated in Fig 3 stimulation with 1/sec for 28 min resulted in a gradual rise in FFA outflow which reached a level of about 0 5 µeq/min 100 g Following the cessation of the stimulation the FFA outflow increased still further before returning towards the prestimulation level *However, the major part (about 60 per cent)* of the net release occurred during the stimulation period The perfusion pressure did not change appreciably during the

play a significant role in the release of FFA under physiologic conditions. The release of FFA is blocked by sympatholytic agents (Rosell 1966). It is, therefore, reasonable to postulate that the sympathetic nerves are of the adrenergic type, with noradrenaline as a transmitter substance. However, Wirsén (1964) was unable to demonstrate adrenergic fibres around the fat cells of rat epididymal and interscapular adipose tissue using the fluorescence method by Falck and Hillarp (Falck *et al* 1962, Carlsson, Falck and Hillarp 1962) which demonstrates noradrenaline in nerve fibres. Later, with improved techniques, adrenergic terminals could be seen between the fat cells in brown adipose tissue. On the other hand, apart from the innervation of precapillary blood vessles, no terminals were revealed in white adipose tissue (Wirsén 1965). In view of the present results the findings of Wirsén are surprising since subcutaneous adipose tissue is of the white type. Species differences or techniqual difficulties in freeze — drying adipose tissue may account for the failure to demonstrate nerve fibres to the fat cells in white adipose tissue with the fluorescence method. Furthermore, there is the possibility that the adrenergic transmitter substance diffuses from the vasomotor nerve terminals to the fat cells. However, details concerning adrenergic innervation of adipose tissue need to be further elucidated.

Even though there is good evidence from the literature (see Introduction) that the sympathetic nervous system, to some extent, regulates the release of FFA from adipose tissue, techniques have been lacking which allow quantitative measurement of the relationship between sympathetic nerve activity and the net release of FFA. Nash *et al* (1961) and Paoletti *et al* (1961) used the omental adipose tissue in dogs to investigate the nervous system regulation of FFA release. They found an enhanced release following nerve stimulation, but their experiments did not yield consistent results and no quantitative data were reported. One explanation for this may be that a high stimulation frequency was applied (16/sec). Under these circumstances the vasoconstriction may have been great enough to trap the released FFA within the tissue (see below). Correll (1963) incubated adipose tissue from rabbits and rats and then stimulated the attached nerve with a very high frequency (50/sec) for 2 hrs which enhanced the release of FFA into the medium. Since the blood circulation was not intact the method as used by Correll may not allow measurements which, from the quantitative point of view, can elucidate the role of the sympathetic nervous system in eliciting the release of FFA.

The present experiments clearly indicate that the vasomotor reactions following sympathetic nerve stimulation interfere to a large extent with the release rate of FFA. At stimulation frequencies above 3/sec an intense vasoconstriction occurs which more or less traps the released material within the tissue. Following cessation of the stimulation the vasoconstriction disappears and may be followed by a vasodilatation. Concomitantly, there is an increased outflow — overflow — of FFA (Fig. 1 and 5). Signs of overflow following cessation of the stimulation may occur (Fig. 3) even at stimulation frequencies within the physiological range. However, there is a difference between the pattern of liberation into the venous blood following stimulation

1—3/sec, and with higher frequencies of say 5/sec, or more In the first case most of the liberated FFA appears in the venous blood during the stimulation period, while in the second case the major part of the FFA occurs after the stimulation period These findings indicate that the concomitant vasoconstriction diminishes the release rate of FFA especially at frequencies considered to be above the physiologic range The mechanism responsible for the diminution of the release rate has not yet been elucidated However, there is reason to suggest that vasoconstrictor nerves may regulate the number of patent capillaries which determine the surface area across which the transcapillary transport of mobilized FFA occurs Such a mechanism would be similar to that found in skeletal muscle (Renkin and Rosell 1962)

Several factors have to be considered for the evaluation of the physiological significance of the nervous release of FFA It should be pointed out that in the present experiments the mixed nerve or the sympathetic chain was stimulated In both cases nerve fibres of different physiological significance may have been stimulated, and there is the possibility that there are specific vasomotor nerves causing vasoconstriction, and specific metabolic nerves responsible for FFA release In this case the vaso constriction, with an impairment of the release rate may not occur when only the hypothetical metabolic nerves are activated In addition, humoral and nutritional factors may also influence the release of FFA following sympathetic nerve activity (see Havel 1965) In order to keep the nutritional factors reasonably constant the dogs were fasted for approximately 24 hrs The existence of these modifying factors may explain the variability in response in different animals

If we disregard the factors which may modify the sympathetic nervous system release of FFA, the experimental data show that a stimulation frequency of 1/sec induces a release rate of about 0 5 μeq/min 100 g adipose tissue while the maximal effect is obtained with a release of 1 5 μeq/min 100 g adipose tissue at 3/sec Provided FFA is taken up by different tissues and completely oxidized in the organism the released amounts correspond to a caloric value of about 1 3 cal/min 100 g adipose tissue at 1/sec and 3 9 cal/min 100 g adipose tissue at 3/sec[1] Since these frequencies are considered to occur under physiologic conditions the data show that sympathetic nerves to subcutaneous adipose tissue have the ability to mobilize energy for the organism within a few minutes

[illegible] by the Swedish Medical Research Council (Project No [illegible] n Anderssons Minne and by Karl Petréns Minnesfond [illegible] advice in setting up the FFA method and Miss Mona Engqvist for skilful help in performing the experiments

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[1] 0 5 μeq FFA ≈ 0 14 mg thus 0 14 × 9 3 = 1 3 cal/min 100 g

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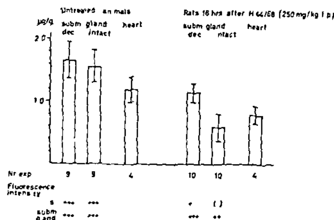


Fig 1 NA in the heart and in the submandibular glands (µg/g of wet weight) of rats decentralized on one side (see methods) Part of the animals were treated with H 44/68 Means ± SD The histochemical fluorescence picture of the organs is indicated semiquantitatively, means a normal fluorescence picture

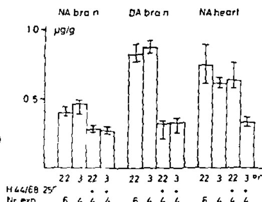


Fig 2 NA and DA in mouse heart and brain (µg/g or wet weight) Means ± SD + substance given — = substance not given (controls)

intact innervation However, in the animals treated with H 44/68 the adrenergic nerves of the intact innervated iris had almost completely lost their fluorescence while in the intact submandibular gland there was a slight but observable reduction In the decentralized organ, on the other hand, no reduction could be found in the gland and only a very slight one in the iris (Fig 1) A slight decrease of the specific fluorescence could be found in the adrenergic nerves in the hearts of the animals treated with H 44/68

There was only small difference between the NA content in the intact and the decentralized submandibular glands in animals not treated with the drug (Fig 1) However, in the animals treated with H 44/68 there was a decrease in the NA content on both sides, but greater on the intact side than on the decentralized one

The estimation of the NA content in the hearts of some rats treated or untreated with H 44/68, showed about the same relative decrease of the transmitter as in the intact innervated submandibular glands (Fig 1)

When mice were exposed to cold for 3 h the catecholamine (CA) content in the heart did not show any marked changes (Fig 2) After 250 mg/kg i p of H 44/68

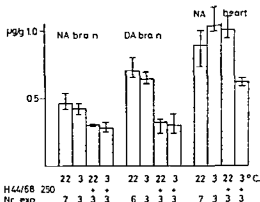


Fig 3 NA and DA in rat heart and brain (µg/g of wet weight) Means ± SD + substance given — = substance not given (controls)

the brain amines were lowered significantly to the same level in animals kept at 22° or 3° In contrast, the concentration of NA in the heart falls significantly only if the mice are exposed to cold In the latter case about 10 per cent of the amine still present is adrenaline, whereas in the other three groups no adrenaline could be detected (*cf* Gordon and Spector 1965)

In rats (Fig 3) the same relationship can be seen However, these animals are much more resistant to cold and, therefore, have to be kept at 3° for 12 h in order to obtain lowered values of NA in the heart

Discussion

It has been shown that the combined effect of nerve stimulation and treatment with an inhibitor of the synthesis of NA causes a depletion of the transmitter from the adrenergic nerve terminals (Malmfors 1964) As it can be seen from the results that after inhibition of the synthesis of NA the NA content in intact adrenergic nerves is decreased more than that in decentralized nerves it can be assumed that the neuronal impulse flow in the former is sufficient to cause a moderate decrease of the NA content after such an inhibition This may be assumed to be the main mechanism of depletion of NA from sympathetically innervated organs after treatment with inhibitors of the synthesis of NA

However there is also a small decrease in the concentration of NA in the decentralized nerves which are supposed to have no neuronal activity This decrease in NA should represent a loss at the uptake sites through the axon membrane (see Malmfors 1965) or an intraneuronal deamination by MAO H 44/68 seems not to affect this uptake of NA into adrenergic nerves Malmfors unpublished results)

Exposing mice or rats to cold stress does not influence the CA content in brain or heart When an inhibitor of tyrosine hydroxylation — the rate limiting step in the biosynthesis of CA — is administered to mice the NA content of the he

within a few hours to low levels compared to that of animals kept at room temperature, whereas there is no difference in the brain amine levels in these two groups This significant decrease of NA at 3° C after the inhibition of transmitter synthesis by H 44/68 shows that this exposure to cold leads to an increased impulse flow in the adrenergic nerves to the heart In these animals the adrenal medulla too seems to be in a state of high activity and a certain amount of adrenaline is released which is taken up from the circulation by adrenergic nerves as it can be demonstrated chemically in the heart Rats appear to be much less sensitive to cold than mice

From these two experiments it is obvious that inhibitors of the synthesis of NA are very useful for functional studies of the activity in adrenergic nerves

The fluorescence method of Falck and Hillarp makes it possible not only to study the cellular and subcellular localization of the biogenic amines but also to obtain a semiquantitative estimation of the relative content of NA in the adrenergic nerves as the fluorescence intensity varies with the NA concentration It may sometimes be difficult to observe moderate changes at high NA levels The histochemical studies show directly that the reduction in the NA content has taken place in the terminals of the adrenergic nerves However, there is a discrepancy in the results from the iris and those obtained from the submandibular gland After inhibition of NA synthesis there is a rather small change in the fluorescence in the intact gland whereas there is a great reduction in that of the intact iris Further there is only a slight alteration in the fluorescence in the decentralized iris Therefore, it seems reasonable to believe that the reduction of the NA content in the iris is greater than that in the submandibular gland Consequently there may be differences in the behaviour of adrenergic nerves towards an inhibition of transmitter synthesis

For a solution of such questions the histochemical method seems to be most suitable

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Acta physiol. scand. 1966. 67. 358—362

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Differences in the Uptake of Secondary Catecholamines by the Adrenergic Nerves

By

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Abstract

Corrodi, H , T. Malmfors and Ch Sachs *Differences in the uptake of secondary catecholamines by the adrenergic nerves* Acta physiol scand 1966 67 358—362

By using the fluorescence method of Falck and Hillarp on whole mounted stretch preparations [illegible]

cats and should be kept in mind when the stimulation potency of these amines on the adrenergic α- or β-receptors is studied The irises from the animals treated with A have furthermore been used to test the reliability of the methods hitherto used for histochemical differentiation between A and NA

Injections of labelled or unlabelled adrenaline (A) or noradrenaline (NA) have been found to cause an accumulation of these amines in sympathetically innervated tissues (Axelrod, Weil-Malherbe and Tomchick 1959, Muscholl 1960, Whitby, Axelrod and Weil-Malherbe 1961, Stromblad and Nickerson 1961, Andén 1964), probably in the sympathetic nerves since there is no accumulation after postganglionic denervation (Hertting *et al* 1961, Stromblad and Nickerson 1961, Anden, Carlsson and Waldeck 1963) With the fluorescence method of Falck and Hillarp for the demonstration of monoamines at the cellular and subcellular level, it has been directly shown that NA is taken up by the adrenergic nerves primarily by a highly efficient mechanism that is not influenced by reserpine and which is in all probability located to the axon membrane (Malmfors 1965) The infusion of NA may temporarily restore the response to electrical stimulation of sympathetic nerves and to tyramine in reserpine-treated animals (Burn and Rand 1958, 1960, Rosell and Sedvall 1961) in which these effe[illegible] been abolished (Carlsson *et al* 1957, Burn and Ran[illegible]

Among secon[illegible] holamines A, [illegible] isoprenaline (IP), has been found

[1] Presented in part [illegible] im on [illegible] April 7 196[illegible]

to restore the sympathomimetic effect of tyramine in isolated hearts from reserpine treated rabbits (Andén *et al* 1964) This could probably be explained by lack of uptake of IP in the isolated rabbit heart

It has furthermore been shown that the positive chronotropic and inotropic effects of IP in isolated rabbit hearts (Brogård Corrodi, Persson, to be published) and in anesthetized mice and cats (Henning, pers comm) could be considerably prolonged by previous inhibition of the catechol O methyl transferase inactivating catecholamines whereas the effects of NA and A on the heart were influenced by such treatment only to a very minor degree These observations lend strong support to the idea that IP cannot be inactivated by uptake into the adrenergic nerve terminals

The aim of the present investigation was to study the uptake of secondary cate cholamines in the adrenergic nerves at cellular level with the fluorescence method of Falck and Hillarp

Material and Methods

Results

In the same way as the primary catecholamines NA dopamine and the correspond ing α methylated derivates are converted into the intensely fluorescent 6 7-dihyd roxy 3 4 dihydroisoquinolines during the treatment with formaldehyde gas A, IP and N isopropyl-dopamine are converted to N substituted 6 7-dihydroxy 3 4 di hydroisoquinolinium compounds which show the same green to yellow green fluor escence in the fluorescence microscope used Corrodi and Hillarp 1963)

In the normal albino rat iris treated with formaldehyde gas the adrenergic nerves display an intense fluorescence emanating from the reaction product formed from the endogenous intraneuronal NA Corrodi and Hillarp 1963 1964 The appearance of the adrenergic innervation in whole mounted stretch preparations of rat iris has been studied in detail in other papers Malmfors 1965 Malmfors and Sachs 1965) The adrenergic nerves form a ground plexus uniformly distributed over the whole iris in the dilator zone the sphincter zone and around some small arterioli The strands of the plexus contain two or more fine fibres running together These fibres which have proved to be the adrenergic terminals have a highly characteristic appearance, with abundant small elongated enlargements or "varicosities which exhibit a very strong fluorescence Besides the terminals there are non axons which have a low fluorescence intensity

TABLE I. The intensity of the restituted fluorescence of the adrenergic nerves in rat iris after the administration of different secondary amines preceded by nialamide to reserpine treated animals as compared to the fluorescence intensity in normal animals. Number of animals in parenthesis

	Dose mg/kg i.v.	Fluorescence intensity	
Adrenaline	0.1	+	(6)
Adrenaline	0.5	++	(4)
Isoprenaline	5	−	(5)
N-isopropyldopamine	5	−	(5)
Normal		+++	

The rat iris is particularly suitable for pharmacological studies with the fluorescence method of Falck and Hillarp, since it is easy to prepare, needs no sectioning, shows the adrenergic nerves in their whole length and provides fairly good possibilities of estimating the fluorescence down to a very low intensity. It has been used in a large scale pharmacological investigation on the adrenergic nerves (Malmfors 1965).

18 hrs after treatment with reserpine (10 mg/kg i.p.) there is no specific fluorescence left and the same is true up to 36 hrs after the injection (Malmfors 1965). Exogenous NA preceded by nialamide, however, restitutes the fluorescence of the adrenergic nerves. The restituted fluorescence has a slightly different appearance from that in the normal animals. The very strongly fluorescent varicosities which are found in the normal animals are lacking and the terminals look smooth. Furthermore the non terminal axons, which have a lower fluorescence intensity than the terminals in the normal animals now have a higher intensity than the terminals (see Malmfors 1965 and microphotos in the same publication). Nialamide alone has no ability or — after a long time — a very slight ability to restitute the specific fluorescence in reserpine treated animals.

In the animals pretreated with reserpine and treated with A preceded by nialamide the specific fluorescence had been restituted in the same way as after NA treatment. The nerves showed the same distribution and appearance. The specific fluorescence after 0.1 mg/kg was weak but clearly visible and after 0.5 mg/kg strong but not as strong as in the normal animals (see Table I). There was no clear difference in fluorescence intensity between the irises that were treated for 1 hr with formaldehyde gas and those which were treated for 3 hrs. l- and d-A gave the same result.

After treatment with IP or N-isopropyl dopamine, on the other hand, no specific fluorescence at all could be found in the adrenergic nerves even after the highest doses as shown in Table I. After these doses, however, there was sometimes a slight increase in the background fluorescence.

Discussion

From the results obtained it is obvious that A like NA can be taken up into the adrenergic nerves by a mechanism not inhibited by reserpine and probably located

in the axon membrane There is consequently no doubt that the A found to be accumulated in intact sympathetically innervated organs (Axelrod, Weil Malherbe and Tomchick 1959) is at least partly taken up by the adrenergic nerves On the other hand IP and N isopropyl dopamine, which in model experiments give the same fluorescence as A after treatment with formaldehyde gas, are not taken up by the adrenergic nerves The increased background fluorescence after treatment with very high doses of IP or N isopropyl dopamine probably represents an unspecific retention of the amines in the tissue This shows also that these N substituted secondary amines give the fluorescence reaction in tissues

The chemical reaction between formaldehyde and secondary catecholamines requires more drastic conditions than that between formaldehyde and primary catecholamines, due to the formation of a quarternary nitrogen (Corrodi and Hillarp 1964, Jonsson, pers comm) This fact has hitherto been used for the histochemical differentiation between NA and A One hr treatment at 80° C has been considered optimal for NA and 3 hrs treatment optimal for A *In the present investigation*, however, there is no clear difference in the fluorescence intensity of the flourophores derived from A after one or 3 hrs reaction at 80° C It is therefore obvious that this is an unsatisfactory way to distinguish between NA and A histochemically Falck and Owman (1965) have described another method for this differentiation They claim that the fluorophore from A can be dissolved in organic solutions such as xylene which do not dissolve the fluorophore from NA When the iris preparations from the animals treated with A in the present investigation were treated with xylene there was no diffusion fading or any other change in fluorescence Similar observations have been made in model experiments (Jonsson, pers comm) It is therefore doubtful whether this dissolving test can be used for histochemical differentiation between NA and A in all cases

The restoration of the response to electrical stimulation of sympathetic nerves and to tyramine in reserpine treated animals by A could be explained by A being taken up into the adrenergic nerves in the same way as NA IP, on the other hand, cannot produce the same effect probably because it is not taken up by the adrenergic nerves

The uptake of NA and also A by the adrenergic nerves has been considered to be a very important inactivation mechanism for these amines in animals (see Axelrod 1964) As IP is not inactivated in the same way and mostly has to be inactivated enzymatically by catechol O methyl transferase its action on the adrenergic receptors differs in duration and strength as compared with that of NA and A This should be kept in mind when studies on the potency of different catecholamines on the adrenergic α or β receptors are performed in isolated organs or in intact animals A direct comparison of the stimulation potency of these amines is only possible if this difference in inactivation mechanisms is avoided by blocking the uptake of NA and A through the axon membrane by an agent like desmethyl imipramine

This investigation has been supported by Research Grants (12X—711—01) from the Swedish Medical Research Council and by a U S Public Health Service Research Grant (NB 05236 01) from the National Institute of Neurological Diseases and Blindness

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Effect of Cervical Sympathetic Stimulation on Accommodation in Monkeys

An example of a beta-adrenergic, inhibitory effect

By

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Abstract

TORNQVIST, G *Effect of cervical sympathetic stimulation on accommodation in monkeys An example of a beta-adrenergic, inhibitory effect* Acta physiol scand 1966 67 363—372

The preganglionic cervical sympathetic nerve, of mainly cynomolgus monkeys (*Macaca irus*) has been [illegible]

elimination of the sympathetic stimulation response, though changes in eye volume seemed uninfluenced The accommodative response (in diopters) to stimulation increased considerably when the eye was under strong cholinergic tone When the animal was pretreated with a large dose of atropine cervical sympathetic stimulation did not affect the refractive state of eye The results indicate the presence of a β adrenergic inhibitory mechanism of the ciliary muscle Possibly this mechanism is responsible for distance accommodation (decrease in accommodation) Changes in vascular structures upon sympathetic stimulation play no practical part in regulating the accommodation

Morat and Doyon (1891) were the first to show that stimulation of the cervical sympathetic nerve inhibited accommodation in the cat, dog and rabbit However, their results were not confirmed by Hess and Heine (1898) or Romer and Dufour (1902) The problem was reviewed in 1937 by Cogan, who mainly on theoretical grounds accepted a dual innervation of the ciliary muscle The Morgan-Olmsted group *stated in several papers Olmsted 1944) that stimulation of the sympathetic* nerves to the eye caused a refractive change in the direction of hypermetropia Whether the sympathetic effect on accommodation is caused by reducing the blood flow through the uvea, as Fleming and Hall states 1959 , or by a direct inhibitory action on the muscle cells has not yet been settled Meesmann enucleated cat and human eyes, 1952) and van Alphen *et al* muscle strips of rabbit, cat, and monkey, 1965) have demonstrated *in vitro* the presence of adrenergic receptors in the ciliary muscle In the rhesus monkey these are only inhibitory β receptors

In vivo accommodation experiments reported in the literature have mostly been performed on lower animals with a narrow range of accommodation. The present paper deals with the influence of cervical sympathetic nerve stimulation on the accommodative mechanism of monkeys. These animals are known to have a wide range of accommodation (Törnqvist 1966). The effect of α- and β-adrenergic receptor antagonists and different degrees of cholinergic background on the accommodative response to sympathetic stimulation is reported.

Materials and Methods

The main species studied was the cynomolgus monkey (*Macaca irus*). 16 animals weighing 1.0 to 3.7 kg were used. Except for one old and two very young animals the monkeys were all young adults.

To see if the results could be reproduced in other species, two adult vervets (*Cercopithecus aethiops*) weighing 5.5 and 5.6 kg and two young baboons (2—2.5 years of age), one *Papio doguera* (7.3 kg) and one *Papio cynocephalus* (6.0 kg) were also examined.

Some of the monkeys were prepared by double iridectomy of one eye, about a month prior to the experimental procedure which made it possible to record the refractive state when miotics were given (Törnqvist 1964). The monkeys were anesthetized with pentobarbital, 30 mg/kg of body weight, intraperitoneally. They were placed supine and kept warm with infrared lamps.

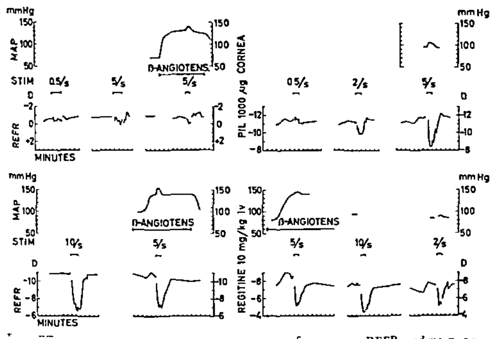
In order to stimulate the sympathetic nerves to the eye, the homolateral cervical sympathetic nerve (preganglionic) was dissected free, as a rule up to the lower end of the superior cervical ganglion.

In all but 4 expts the sympathetic nerve was intact during all measurements. However, cutting the nerve proximal to the stimulation point did not change the response. The electrodes consisted of silver wire (0.5 mm), insulated except for 2 mm at their ends. These were pulled through the walls of a larger plastic tube, which had a longitudinal slit cut along one side. The nerve was in- [illegible]

The refractive state, usually of the horizontal meridian, was read from a Thorner optometer (Thorner 1927), which gave the axial spectacle plane refraction in diopters (D). The optometer was controlled with trial lenses and showed errors less than ± 0.25 D. The refractive state was followed almost continuously during the sympathetic nerve stimulation and during the minutes just before and after stimulation. As a rule 10 to 16 readings were obtained during a stimulation period of 1 min. To make the refractive readings more exact, especially in experiments where the eyes were punctured, a corneal contact lens was often used (G. Nissel & Co. Ltd., London). The spherical lens had an inner curvature of 6.0 mm, the power was — 5.0 D, its axial thickness 0.12 mm and the overall diameter was 8.0 mm. The same lens was used for all monkeys. Thus the fit to the corneal surface was not perfect in every case. Therefore the shift in dioptric power when the lens was applied to the eye sometimes differed from 5 D. It can be deduced theoretically that a constant refractive change inside the eye gives a slightly larger response, measured as change in spectacle plane refraction, if the contact lens is present.

The results are shown in time response diagrams. To obtain these, consecutive refraction readings were grouped together 2 and 2 and averaged. The means were plotted against time in minutes and connected by straight lines (Fig. 1 to 4).

In some experiments the intraocular volume change during stimulation was also recorded. This was accomplished by connecting the anterior chamber to a small polyethylene container with perfusion fluid, the weight of which was constantly recorded by means of a force transducer (Bárány 1962). With this arrangement rapid volume changes as small as 0.5 μl could be recorded. The height of the perfusion fluid container was adjusted to give an inflow into the eye of about 5 μl/min, which occurred at an intraocular pressure of 15—25 mm Hg. The perfusion fluid used was that described by Bárány (1964). [illegible] femoral artery and connected to a pressure transducer.



ulations β angiotensin II 0.5 μg/min was infused. A corneal contact lens was used in the 8 last stimulations

The effect of sympathetic stimulation on accommodation was studied at three levels of cholinergic background

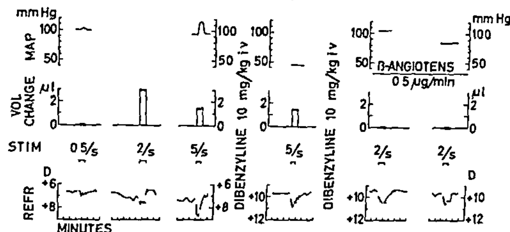
Propranolol (Inderal[R] ICI) which has recently been introduced (Black *et al.* 1964) was the only

Drugs. The amounts of the following drugs refer to their salts: pentobarbital [illegible] (hydrochloride), eserine (salicylate), atropine (sulfate), Regitine methanesulfonate, Dibenzyline (hydrochloride) and propranolol (hydrochloride). The amounts of β angiotensin II refer to the free compound. Systemic doses are in mg/kg body weight.

Results

General observations

All monkeys examined responded with a hypermetropic change in refraction during stimulation of the cervical sympathetic nerve. An effect was usually recorded in



two stimulations 105 and 120 min after the first and 50 and 65 min after the second Dibenzyline dose A corneal contact lens was used during the whole experiment

less than 10 sec after beginning of stimulation In experiments where miotics were applied onto the cornea maximal responses were reached within 20 to 40 sec However, the apparatus was not designed for a close study of the time-response relationships A stimulation frequency of 0 5/sec gave little or no response A frequency of 2/sec always caused a distinct decrease in accommodation The effect increased up to 10/sec but a further increase in stimulation rate from 10 to 20/sec usually gave little or no further augmentation of the response

In two monkeys the refractive state was followed both in a horizontal and a vertical meridian during sympathetic stimulation No distinct difference in response was seen between the meridians

A very distinct feature which is obvious in Fig 1 was that the effect of sympathetic stimulation was considerably increased after the eye had been made myopic by drugs (pilocarpine or eserine) In 6 eyes tested before and after a miotic was given onto the cornea the same stimulation frequencies gave responses which were 2 to 4 times *greater* (expressed in D), when the cholinergic background was artificially increased

All cynomolgus monkeys except two, showed an increase in mean arterial pressure (usually only a few mm Hg) when stimulated with 0 5 to 10 stimuli/sec This effect was independent of corneal treatment with parasympathomimetics When a fall in blood pressure occurred this was small and of no importance

Effect of adrenergic α-receptor antagonists on eyes under the influence of pilocarpine or eserine given locally

Two cynomolgus monkeys were given Regitine i v , one 5 mg/kg and the other 10 mg/kg (Fig 1) Cervical sympathetic stimulation caused about the same hyper-

TABLE I Effect of adrenergic blocking drugs on volume change (i e the amount of fluid going into the eye) at different frequencies of cervical sympathetic stimulation Cynomolgus monkey no 1 was given 10 mg/kg of Dibenzyline (phenoxybenzamine) No 2 was given 10 mg/kg before the 5/sec stimulation was performed and another 10 mg/kg before the 2/sec stimulations Monkeys no 3 and 4 were given 2 mg/kg and no 5 was given 4 mg/kg of propranolol (Inderal)

	Dibenzyline				Propranolol					
		Stim/sec				Stim/sec				
	Monkey	0.5	2	5	Monkey	0.5	1	2	5	10
Before	Cynom no 1	0	4.5	4.5	Cynom no 3	0				1.5
	Cynom no 2	0	3	1.5	Cynom no 4	0	1	0	2	2
					Cynom no 5	5	5		7.5	9
After	Cynom no 1		1	0	Cynom no 3		0		1	
	Cynom no 2		{0, 0, 0}	1.5	Cynom no 4				{1, 1.5}	
					Cynom no 5				7.5*	

* = mean of 5 recordings

metropic change after Regitine as before the drug was given, although lid fissure widening and piloerection were greatly diminished

In two other cynomolgus monkeys the intraocular volume change induced by sympathetic stimulation was measured at the same time as the refractive state, before and after Dibenzyline (10 and 20 mg/kg i v) No definite decrease in refractive change could be observed in the monkeys after the α adrenergic blocking drug (Fig 2), though the drug caused a distinct fall in blood pressure (from 70—100 to 40—50 mm Hg) This did not change upon sympathetic stimulation Restoring the blood pressure with β angiotensin II neither increased nor decreased the effect of stimulation The volume changes of the eyes are shown in Table I After Dibenzyline cynomolgus monkeys number 1 and 2 responded when stimulated with much smaller volume changes

Effect of an adrenergic β receptor antagonist on eyes under the influence of pilocarpine or eserine given locally

Four cynomolgus monkeys were given propranolol i v after eserine or pilocarpine had been applied onto their cornea This drug caused an immediate and considerable fall in heart rate (Fig 3 though the blood pressure remained grossly unaffected In three of these monkeys 2 mg kg propranolol was sufficient to prevent the accommodative response to sympathetic stimulation Fig 3 in the fourth monkey, 4 mg/kg was needed In 3 of these monkeys eye volume changes were measured simultaneously with the refractive state Table I The volume changes upon stimulation

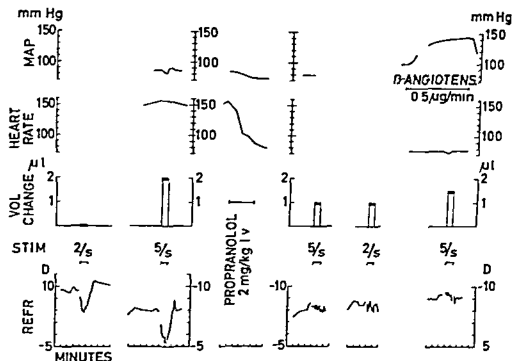


Fig 3 Effect of cervical sympathetic nerve stimulation on refractive state (REFR) eye volume (VOL CHANGE) heart rate and mean arterial blood pressure (MAP) before and after intravenous propranolol (Inderal) One mg of pilocarpine was applied onto the cornea before the first stimulation. The last three stimulations were performed 20 35 and 45 min after propranolol A corneal contact lens was used during the whole experiment

seemed to be unaffected by propranolol, while the refractive changes vanished In two other non iridectomized cynomolgus monkeys with no miotic given, propranolol 2 mg kg also eliminated the hypermetropic responses to stimulation On the other hand, the pupil dilated and the lid fissure widened just as much after, as before the β receptor antagonist was administered — The experiments on the two baboons and on the two vervets indicate that in these species, too, the refractive changes to sympathetic stimulation are blocked by β receptor antagonists but are unaffected by α receptor antagonists

Effect of atropine

As mentioned previously the effect of sympathetic stimulation on refraction was distinctly increased if the eye was under increased cholinergic tone (Fig 1 This fact demanded an examination of the effect of sympathetic stimulation on the refractive state when the endogenous cholinergic tone had been antagonized by atropine As no local parasympathomimetic was given this part of the investigation was performed on eyes which had not been iridectomized and thus allowed a simultaneous study of the pupil Atropine, 1 0 mg kg, gave a wide pupil and extinguished the pupillary light reflex in the 4 cynomolgus monkeys used Prior to atropine all monkeys showed small but definite, hypermetropic changes in refraction on sym

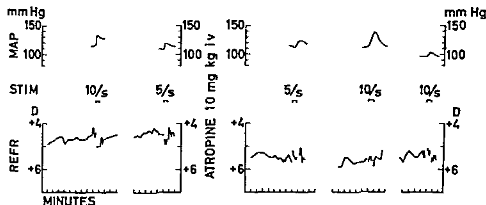


Fig 4 Effect of cervical sympathetic nerve stimulation on refractive state (REFR) and mean arterial blood pressure (MAP) before and after i v atropine No pilocarpine was given. The last 3 stimulations were performed 30 50 and 65 min after the atropine A corneal contact lens was used during the whole experiment

pathetic nerve stimulation (Fig 4) After atropine no refractive changes were seen on stimulation although the pupils widened distinctly

Effect of stimulation on eye position

Of the 5 monkeys tested, 3 showed no detectable retraction or protrusion of the bulb on sympathetic stimulation (5 to 10 stimuli/sec) neither before nor after pilocarpine or eserine was applied onto the cornea One eye retracted 0 5 to 0 1 mm three times upon stimulation (10 stimuli/sec), whereas another moved inward 0 05 mm once and outward 0 05 mm twice — Thus, the shifts in eye position during stimulation were very small and inconsistent

Discussion

In the earlier *in vivo* experiments where accommodation has been studied during sympathetic stimulation, the stimulation frequencies used were high In the present paper different frequencies within what is considered physiologic limits were employed (Folkow 1952, Hillarp 1960) This must be a pre requisite in estimating the physiologic role of the sympathetic innervation to the ciliary muscle

Another condition to which attention must be paid when performing *in vivo* experiments is the state of blood circulation in the animals examined This becomes very important when as in this investigation drugs affecting the whole vascular system are used A decrease in blood pressure can cause a cholinergically induced accommodation to vanish Tornqvist unpublished data To keep blood pressure at average or high levels after administration of α receptor antagonists angiotensin was infused It had *per se* no effect on the refractive state in non accommodating, normotensive monkeys Given to monkeys whose eyes are under increased cholinergic tone, it increases accommodation when blood pressure is low This is ascribed to its re establishing effect on the blood flow through the eye Tornqvist unpublished data) Anyhow, the hypermetropic refractive change on sympathetic stimul

remained the same after adrenergic *a* receptor antagonists had been given, whether angiotensin was infused or not

Whether the sympathetic nerves act directly on the ciliary muscle or by means of changing the volume of the vascular bed has been discussed since Morat and Doyon (1891) These authors found retinal vasodilatation during stimulation in the dog and cat and vasoconstriction in the rabbit and therefore thought that there was no vascular influence on accommodation, since all three species gave similar lens changes on stimulation Fleming and Hall (1959) on the basis of a histophysiological technique in cats proposed that the change in volume of the ciliary body induced by the sympathetic nerves is the main mechanism by which accommodation is influenced

On the other hand, recent histochemical studies by the Hillarp Falck technique reveal close terminal connections between adrenergic nerve fibers and ciliary muscle cells in the monkey (Ehinger 1966) van Alphen *et al* (1965) have also shown that sympathetic amines *in vitro* relaxed monkey ciliary muscle strips and that this relaxation could be blocked by dichloroisoproterenol (DCI) which is a adrenergic *β* receptor antagonist

For several reasons the present *in vivo* investigation demonstrates that the sympathetic nervous system relaxes accommodation not by influencing the vascular tone of the ciliary body, but by affecting the muscle itself Firstly, the adrenergic *a* receptor antagonists did not decrease the accommodative response on sympathetic stimulation though the volume changes decreased considerably Casey (1966) found much lesser changes in eye volume on monkeys on stimulation after Regitine 10 mg/kg) than before the drug That the volume change is a vascular effect is reasonable though not proven In the cat and rabbit Bill (1962) has shown the vascular receptors to be of *a* type Secondly, the adrenergic *β* receptor antagonist did abolish the accommodative response though the volume changes remained A third finding that makes changes in the eye circulation improbable as a cause of the accommodative changes is that the accommodative response on sympathetic stimulation vanished after atropine However, mechanical factors in the lens may be responsible for this lack of effect (see later)

Cholinergic fibers in sympathetic nerves have been described e g by Bacq and Fredericq (1935, nictitating membrane of cat) and by Folkow and Uvnas 1950 vasodilator fibers to skeletal muscles of the cat) Atropine reduced the response to sympathetic nerve stimulation in those investigations It is however not probable that cholinergic fibers are responsible for the hypermetropic response to sympathetic stimulation, because it vanished after the adrenergic *β* receptor antagonist propranolol) was given This drug had no anticholinergic effect on accommodation

That propranolol should block the stimulated nerve at some place preterminally is improbable since the pupil and lid fissure dilated on stimulation also after propranolol It thus seems very probable that the sympathetic system affects the accommodation essentially over an adrenergic *β* receptor

Could it be that the effect on accommodation depended on overflow of the *a*

transmitter (from vascular structures) to β receptors? This is very improbable for several reasons The eye volume measurements indicate that change in volume and accommodation occurred at the same stimulation frequencies and, besides, these frequencies are the same as described for uveal vascular effects, in cat and rabbit (Bill 1962) Also, if there is a transmitter overflow, the response to stimulation after Dibenzyline should have been increased when compared to the values before the α blockade (Brown 1960)

The proof that β adrenergic actions can arise from sympathetic stimulations has become possible only recently with the advent of the β adrenergic blockers It seems as if the ciliary muscle can be grouped with the few known systems, where a sympathetic nerve stimulation causes β-adrenergic action (heart Moran and Perkins 1958, bronchial tree de la Mata *et al* 1962) In the primate ciliary muscle, the β effect is the important one

In the present paper the effect of sympathetic nerve stimulation has been investigated at three arbitrary levels of cholinergic background no cholinergic tone, a small (endogenous) and a greatly increased tone No effort has yet been undertaken to see how different degrees of greatly increased tone influence the stimulation response

The pioneers of research on sympathetic influence on accommodation (Morat and Doyon 1891) found it easier to show the effects when the eye was under increased cholinergic tone Later investigators mostly ignored this problem It is touched upon by Biggs *et al* (1959) who found that subconjunctivally injected epinephrine gave a greater effect at the near point than at the far point of the human eye The explanation may lie in the mechanical factors proposed by these authors, *i e* the lens cannot flatten more than to a certain degree, but it is also possible that the explanation is a neurophysiological one Suppose, hypothetically, a strong excitatory α-adrenergic innervation of some fibers of the ciliary muscle Also assume these muscle fibers to flatten the lens when contracting and that they are opposed to the cholinergic fibers It is quite reasonable that the sympathetic stimulation would then cause a hyperopic refractive change when the eye is atropinized However, this was not the case in the present investigation The β-adrenergic nerves, on the other hand, which are generally considered to have inhibiting properties, would have nothing to inhibit in an atropinized eye and consequently, stimulation of β adrenergic nerves would give no refractive response

The physiologic importance of the sympathetic innervation to the ciliary muscle is difficult to estimate To state as has been done in some ophthalmologic literature, that the influence of the parasympathetic system on accommodation is so or so many times greater than that of the sympathetic, is meaningless at least in primates, because the adrenergic influence depends on the degree of cholinergic activity

In lower animals *e g* the cat rather great accommodative responses have occurred on sympathetic nerve stimulation (Olmsted 1944) even without an increased cholinergic background It is possible that in these animals an active (for instance α adrenergic) mechanism is responsible for distance accommodation Such a hypo-

thetical mechanism has in the present paper been shown to be of no practical importance in monkeys, where on the contrary an inhibiting β adrenergic system is a possible mechanism by which distance accommodation (decrease in accommodation) is accomplished

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The Effect of DOPA on the Spinal Cord.

1. Influence on Transmission from Primary Afferents

By

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Abstract

Anden, N.-E., M. G. M. Jukes, A. Lundberg and L. Vyklicky. *The effect of DOPA on the spinal cord. 1. Influence on transmission from primary afferents.* Acta physiol. scand. 1966. 67. 373—386.

In unanesthetized spinal cats an i.v. injection of DOPA depresses transmission of short latency

to long lasting activity in the spinal cord and do not depend upon a peripheral loop. The selective action of DOPA shows that its action on transmission in the spinal cord is not secondary to circulatory changes. The working hypothesis is forwarded that DOPA inhibits transmission of short latency effects from the FRA through liberation of transmitter from a descending noradrenergic pathway.

Carlsson *et al.* (1964) employed the histochemical fluorescence technique (*cf.* Hillarp, Dahlström and Fuxe 1965) to demonstrate monoaminergic nerve terminals in the spinal cord. Noradrenaline (NA) and 5-hydroxytryptamine (5-HT) occur in different terminals and both types of terminals seem to belong to descending pathways since the fluorescence in the terminals disappears some days after transection of the spinal cord. Furthermore no fluorescent cell bodies have been observed in the spinal cord (Carlsson *et al.* 1964, Dahlstrom and Fuxe 1965) and biochemical investigations have shown that caudal to a transection NA and 5-HT almost completely disappear from the spinal cord after about 7 days (Magnusson and Rosengren 1963, Carlsson, Magnusson and Rosengren 1963, Anden, Haggendal *et al.* 1964). Less is known about the enzymes, but it has been found that most of the 1-3,4-dihydroxyphenylala-

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nine/5 hydroxytryptophan decarboxylase activity caudal to a transection disappears at about the same rate as the NA and 5 HT (Andén, Magnusson and Rosengren 1965) Further histochemical investigations have shown that the monoaminergic terminals belong to bulbospinal pathways, the cell bodies being located in the medulla *oblongata* and lower pons (Dahlstrom and Fuxe 1965) Since there is a dense monoaminergic termination in the intermedio-lateral column in the spinal cord some of these pathways very likely belong to the autonomic nervous system There is, however, also a scattered monoaminergic termination in other parts of the grey matter which may belong to pathways with other functions

It is more difficult to investigate the function of monoaminergic pathways in the central nervous system than in the peripheral autonomic system, one reason being that the monoamines do not pass the blood brain barrier (Fuxe and Hillarp 1964) However, some amino acids acting as intermediates in the synthesis of these mono amines do pass this barrier This is true for both the catecholamine precursor DOPA (l-3,4 dihydroxyphenylalanine) and the 5 HT precursor 5 HTP (5 hydroxytryptophan) These precursors are assumed to enter the nerve terminals and give increased synthesis and "overflow" of the transmitters It was first shown by Euler and Uddén (1951) that NA is formed from DOPA in peripheral adrenergic neurones DOPA gives a formation of DA (dopamine) and NA also in the CNS (Carlsson *et al* 1958) and 5 HTP a formation of 5 HT (Udenfriend, Weissbach and Bogdanski 1957) Histochemical experiments have shown that after inhibition of the monoamine oxidase, DOPA and 5-HTP give an increased fluorescence in NA and 5-HT terminals respectively (Fuxe 1965 a, b)

The basis for the present investigation was the finding by Carlsson *et al* (1963) that DOPA and 5 HTP increase the flexor reflex in the acute spinal animal This suggested the possibility that monoaminergic descending pathways are concerned with the control of somatic reflexes, which was of special interest in relation to previous investigations on the descending control of spinal reflex pathways (*cf* Lundberg 1964 b, 1966) The present paper describes the effect of intravenous injections of DOPA on transmission in neuronal paths to motoneurones, primary afferents and ascending spinal tracts It will be shown that DOPA has a strong inhibitory effect on transmission from single volleys in the FRA (flexor reflex afferents) and that, despite this inhibitory effect, the flexor reflex may be increased after DOPA The problem whether DOPA, as outlined above, acts via a descending NA pathway will be dealt with in a separate paper (Anden, Jukes and Lundberg 1966) Some of the present results were briefly reported (Anden *et al* 1963, Andén, Jukes *et al* 1964)

Methods

All experiments were made on unanesthetized spinal cats In a few experiments they were decerebrated by intercollicular sections but this preparation was unsuitable because the high blood pressure after DOPA often produced intracranial bleedings In most experiments we used the anaemic decorticate preparation (Voorhoeve 1960) with the following modification in the procedure At the end of the operation all carotid branches were ligated and the level of ether anesthesia somewhat decreased The preparation is accepted if at this stage extensor rigidity develops if not the basilar artery is exposed and clipped at a mid pontine level Control experiments have shown that in cats

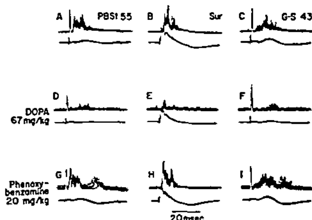
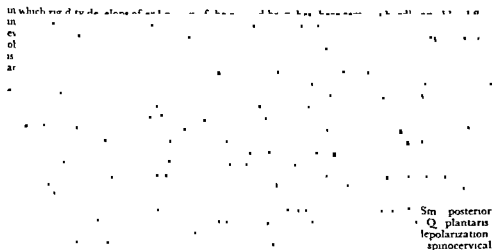


Fig 1 The effect of DOPA on ventral root discharges evoked from primary afferents The upper traces are recorded from the S_1 ventral root and the lower from the L7 dorsal root entry zone A C were recorded immediately before, D F 5 min after the injection of DOPA and G-I 15 min later, a few minutes after the injection of phenoxybenzamine In each column the strength of stimulation is constant and indicated above in multiples of threshold for the PBSt and G-S nerves All records consist of 3—4 superimposed traces



Results

1) *Transmission to motoneurones*

Fig 1 illustrates the inhibitory effect of DOPA on the ventral root discharges evoked from the FRA The polysynaptic discharges evoked from cutaneous afferents (B) and from high threshold muscle afferents (A, C) are very effectively depressed after iv injection of DOPA (D F) The lower row of records shows that the effect of DOPA is entirely reversed by the α receptor blocker phenoxybenzamine There is only a relatively small decrease of the flexor Ia monosynaptic reflex in D (cf also below) and this suggests that the depression of the polysynaptic discharges from the FRA is not caused by a reduced excitability in motoneurones but mainly by an inhibition of transmission in the pathway from the FRA to motoneurones This problem has been further investigated by the technique of conditioning monosynaptic reflexes and

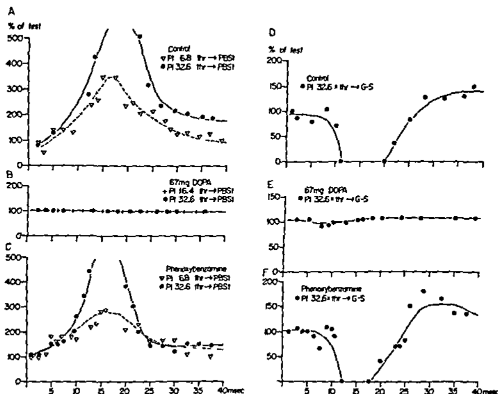
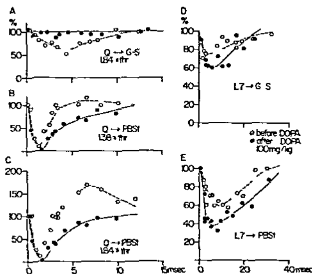


Fig 2 The effect of DOPA on synaptic action from high threshold muscle afferents in motoneurones The curve shows the effect of single conditioning volleys on the monosynaptic test reflexes from PBSt (left curves A-C) and G S (right curves D F) 100 % on the ordinate is the unconditioned test [illegible] after DOPA and 5 min after the injection of phenoxybenzamine

Fig 2 shows that the facilitation of flexor and inhibition of extensor test reflexes, normally evoked from high threshold muscle afferents, is abolished after DOPA Hence DOPA inhibits both the excitatory and the inhibitory pathway from the FRA *The lower curves of Fig 2 show the reversal by phenoxybenzamine of this inhibitory* effect

Similar experiments have shown that DOPA also inhibits transmission of synaptic actions from Ib afferents to motoneurones B and C, Fig 3 shows the effect of conditioning volleys in the Q nerve on the PBSt test reflex The decay of the inhibitory curve is much slower after DOPA than in the control curves which suggests that DOPA inhibits the excitatory path from Ib afferents to flexor motoneurones Curve A illustrates the inhibitory effect by DOPA on the inhibitory pathway from Ib afferents to extensor motoneurones There was, on the other hand, no indication that DOPA affects the pathway from Ia afferents to motoneurones Curves B and C, Fig 3 show that *the magnitude of the reciprocal* Ia inhibition is unchanged after

Fig 3 The effect of DOPA on the Ia Ib and recurrent collateral pathways to motoneurones Monosynaptic test reflexes from PBSt and G S were recorded in the SI ventral root In curves A C conditioning volleys were evoked in the Q nerve the strengths are indicated in multiples of threshold A maximal group I volley from Q was evoked at 1 92 times threshold The inhibitory effect in B and C is evoked from Ia afferents and the excitatory effects in these curves from Ib afferents The inhibitory effect in A is produced from Ib afferents The inhibitory effect (Renshaw inhibition) in D and E are produced by single conditioning antidromic volleys in the L7 ventral roots The curves were obtained as indicated before (○) and after (●) injection of DOPA



DOPA Evidence that there is no effect on the Ia excitatory pathway was obtained in experiments where the Ia focal potential, recorded extracellularly in the motor nucleus (G S and PBSt) was found to be unchanged after DOPA (100 mg/kg) The slight decrease of the flexor test reflex and corresponding increase of the extensor test reflex in Fig 1 may be secondary to the inhibition of the FRA pathway, which could result in a withdrawal of a synaptic bombardment excitatory to flexor and inhibitory to extensor motoneurones However, those effects on monosynaptic test reflexes were not consistantly found, in some experiments there was no effect and in others a slight increase or decrease of both extensor and flexor monosynaptic test reflexes These results cannot be taken to indicate that DOPA changes the excitability of motoneurones

The effect of DOPA was also tested on the recurrent collateral inhibitory pathways from motoneurones and curves D and E of Fig 3 illustrate the consistent finding that the Renshaw inhibition (in flexor and extensor nuclei increases after DOPA

The effects described above and in the following section were maximal a few minutes after the injection of DOPA and there was complete recovery in 75—120 min (Fig 6) An injection of DOPA raises the plasma level of catecholamines but the blood brain barrier prevents their entry into the spinal cord The selective effect on transmission makes it very unlikely that the effect of DOPA is secondary to circulatory changes caused by the catecholamines in the blood plasma DA does not pass the blood brain barrier but produces similar peripheral effects as DOPA An injection of DA in a dose 20—50 μg kg min chosen to give the same blood pressure rise as 100 mg kg of DOPA had no effect on transmission from the FRA to motoneurones, primary afferents and ascending spinal pathways

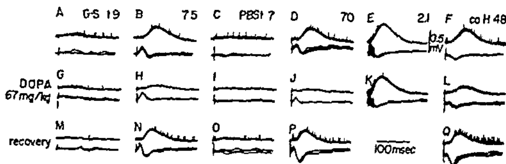


Fig 4 The effect of DOPA on the DRPs Record ng of DRPs (upper traces) was made from the

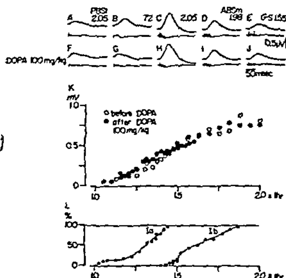


Fig 5 Lack of effect by DOPA on DRPs evoked from group I muscle afferents Records of DRPs before and after DOPA as in Fig 4 The PBSt nerve was stimulated to obtain the graphs In the upper graph the height of the DRP is plotted in mV as a function of the stimulus strength before and after DOPA as indicated The corresponding lower graph (with the same abscissa as the upper) shows the size of the Ia and Ib incoming volley plotted against stimulus strength After DOPA there is slight increase in the height of the DRP evoked by a single group I volley (F) and possibly a slightly increased slope of the curve over its early portion but these small changes are probably not significant Same time scale for all record ngs the vol age scale applies to all DRPs

2) *Transmission to primary afferents*

The effect of DOPA was investigated on the DRPs evoked from different afferent systems There is an effective depression of the DRPs evoked from the FRA as is illustrated for high threshold muscle afferents in Fig 4 and for cutaneous afferents in Fig 6 Hence DOPA depresses transmission from the FRA to ipsilateral primary afferents In L Fig 4 there is also a depression of transmission from contralateral high threshold muscle afferents the fact that this depression is less marked than from the ipsilateral nerves is fortuitous usually DOPA is equally effective in depressing transmission from the contralateral and the ipsilateral FRA There is no effect on the DRP evoked from group I muscle afferents in E and K Fig 4 and this is further illustrated in Fig 5 with the group I DRP from both flexor and extensor nerves The curves in Fig 5 in which the size of the DRP is plotted against the incoming

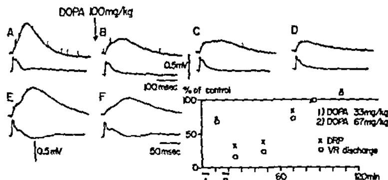


Fig 6 Effect of DOPA … DRP …

DRPs

group Ia and Ib volley, show no significant difference before and after DOPA and this strongly suggests that the lack of effect on the DRP evoked by a train of maximal group I volleys is not due to a stronger synaptic linkage in the pathway from the group I afferents It is concluded that DOPA does not depress transmission from the Ia and Ib afferents of flexors to Ia afferents nor from Ib afferents of flexors and extensors to Ib and cutaneous afferents (Eccles 1964)

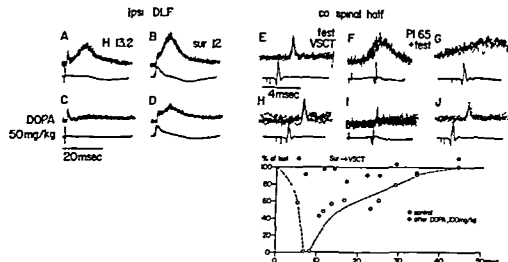
Somewhat conflicting results were obtained with the DRPs evoked from cutaneous afferents The second component which is part of the FRA response (Carpenter *et al* 1963) was always depressed However, the early component I was sometimes as effectively depressed as component II (F, Fig 6) but in other experiments there was only a slight effect (A-D) Component I of the cutaneous DRP represents activity in a pathway from cutaneous to cutaneous afferents (Carpenter *et al* 1963), which is not part of the FRA effect

In Fig 4—6 the traces recorded from the dorsal root entry zone show the expected decrease in the P wave, which is associated with the DRP evoked from the FRA (Barron and Matthews 1938) In H and J Fig 4 this occurs without much reduction in the height of the late negative cord dorsum potent…

…cutaneous afferents, but an effect at this level cannot be entirely excluded

3) *Transmission to ascending pathways*

The effect of DOPA on transmission to ascending spinal pathways is illustrated i… Fig 7 showing recordings from the ipsilateral dissected dorsal part of the lat…



traces Time scale of C also applies to A, B D F and I that of E also to G H and J

funicle (DLF) in A D and from the contralateral spinal half in E-J DOPA has no effect on transmission of the monosynaptic action of group I muscle afferents on DSCT (early component in A and C) or on VSCT (E and H) and no effect on the action of cutaneous afferents on SCT (early component in B and D) On the other hand there is a profound depression of late discharges from high threshold muscle afferents and cutaneous afferents in both the ipsilateral DLF and the contralateral spinal half (F and I) The former discharges are in the DSCT and the SCT the latter discharges mainly in ventral spinobulbar pathways (for reference to ascending spinal pathways cf Lundberg 1964 a, Oscarsson 1965 G and J and the curve in Fig 7 show that there is also an inhibition of transmission of inhibitory action from the FRA to the VSCT It is concluded that DOPA inhibits transmission of both excitatory and inhibitory actions from the FRA to the above pathways

4) *The increase of the flexor reflex*

Carlsson *et al* (1963 found that DOPA increases the flexor reflex evoked by pinching the skin It was therefore surprising to find that DOPA effectively inhibits

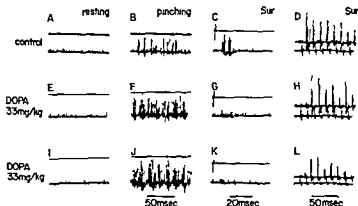


Fig 8 Discharges evoked in efferents to flexors after DOPA Lower traces in the three left hand columns and upper traces in the right hand column were recorded from the central end of the tenuissimus nerve The other trace in each record was recorded from the sciatic nerve A E I are [illegible]

the effects of single volleys in the FRA However, DOPA also depresses the inhibition evoked from the FRA and could thereby remove an inhibitory factor controlling reflex transmission (cf Eccles 1964) Since the effect of DOPA is often more marked on transmission to primary afferents than on transmission to motoneurones the following possibility arose In the acute spinal cat a barrage of impulses in the FRA may maintain a tonic PAD in the FRA giving presynaptic inhibition of transmission of the actions of the FRA to motoneurones It is possible that DOPA by its effective inhibition of the path from the FRA to primary afferents could remove this presynaptic inhibition and thus increase transmission to motoneurones from the FRA, despite the fact that this latter pathway is also inhibited by DOPA In order to test this working hypothesis experiments were made on acute spinal cats in which only the nerve to tenuissimus and the sural nerve were cut Under these conditions DOPA increases the reflex discharge evoked from pinching of the toes (Fig 8, B F, J) but nevertheless there is a depression of the effects of single volleys in the sural nerve (C, G, K) Hence the hypothesis outlined above can be discarded Another explanation of the increased flexor reflex is excluded by records D H and L Fig 8 When a train of volleys is evoked in the FRA a reduced transmission of the later volleys would be expected because of the PAD evoked from the FRA and it had to be considered if it was possible that DOPA by removing this inhibition could enhance transmission to motoneurones of a train of volleys in the FRA However a comparison of D and L Fig 8 immediately reveals that DOPA also reduces the effects of a train of volleys in the FRA Whatever role presynaptic inhibition of the FRA may normally play it seems clear that its removal after DOPA cannot compensate for the inhibitory effect that DOPA has on transmission from the FRA to motone

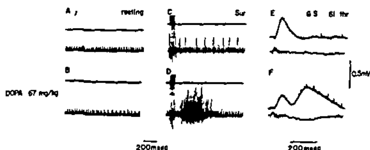


Fig 9 Late actions evoked from the FRA in motoneurones and primary afferents after DOPA In A D records as in Fig 8 from the tenuissimus nerve (lower) and the sciatic nerve (upper) In E and F the upper traces are from the caudal dorsal rootlet in L6 and the lower traces from the L7 dorsal root entry zone A short train of volleys is given in the Sur nerve in C and D and in the G S nerve in E and F A C E were taken before and B D F after injection of DOPA The late DRP in F is evoked from high threshold muscle afferents Observe that the late discharge in D is in both α and γ efferents the discharge in the latter is well seen towards the tail end of the discharge Voltage calibration on the right refers to the DRPs Single traces

However, Fig 9 illustrates a phenomenon that occurs after DOPA and provides an explanation for the increased flexor reflex A D show records made from the nerve to tenuissimus, A and B show resting discharges in γ efferents, C and D show the discharges evoked by a train of volleys in the sural nerve before and after DOPA In the control record C there is an early reflex discharge followed by an after discharge in both α and γ efferents, which lasts throughout the duration of the sweep After DOPA has been given there is a depression of the early reflex discharge but a massive discharge appears after a latency of about 200 msec The duration of the discharge shown in D exceeds 0 5 sec and the discharge occurs in both α and γ efferents A similar discharge was evoked from high threshold muscle and joint afferents but there was never any corresponding effect from group I muscle afferents No late reflex effect was evoked from ipsilateral nerves in efferents to the extensor G S However, volleys in contralateral cutaneous and high threshold muscle afferents produce a late long lasting reflex discharge in α and γ efferents to extensor but not to flexor muscles (Jankowska *et al* 1965) After DOPA a late effect is also evoked in primary afferents as is indicated by the second wave of the DRP in F The late DRP is evoked by the same volleys that give the late discharge in motoneurones i e cutaneous afferents and high threshold muscle and joint afferents

The long latency for the motoneuronal discharge and the DRP made it necessary to investigate whether they depended on a peripheral loop For example if there is an increased γ discharge after DOPA (in D Fig 9 this possibly occurs after a shorter latency than the 200 msec given above) late central actions might be evoked via spindle afferents if neuromuscular transmission to the intrafusal fibres was not blocked Flaxedil was given in all experiments but it is known that neuromuscular transmission from γ-efferents is more resistant to the blocking effect of Flaxedil (for references see Matthews 1964) However, when Flaxedil was given in a dose 5 times that required to block completely transmission to extrafusal muscle fibres there was

no change in the late discharge or the late DRP, hence these effects are not evoked via the γ loop

Afferent discharges were recorded from the dorsal root filaments (L5 or L6) in cats that had received smaller doses of Flaxedil In no case did volleys in the FRA produce an afferent discharge after DOPA had been given Furthermore in other experiments the late effect after DOPA persisted after complete ipsilateral deafferentation The spinal transection was in L2, all ipsilateral dorsal roots caudal to this level except L7 and S1 were cut and the muscles and skin supplied from the two latter segments completely denervated Nevertheless volleys in the FRA produced the late effect These findings seem to exclude the possibility that the late effects evoked from the FRA after DOPA are caused by efferent impulses giving a discharge in afferents from other receptors than muscle spindles It is therefore suggested that the long latency is caused mainly by a long central delay

Discussion

An intravenous injection of DOPA gives a profound inhibition of transmission from the FRA to motoneurones, primary afferents and ascending pathways The inhibitory and excitatory paths from the FRA to motoneurones and ascending pathways are depressed with equal effectiveness DOPA also inhibits trasmission in the excitatory and inhibitory paths from Ib afferents to motoneurones, but there is no effect on transmission in the Ia inhibitory pathway or from either Ib or Ia afferents to primary afferents and to ascending pathways It is extremely unlikely that the absence of effect on some pathways is due to a high safety factor of transmission in those pathways and the evidence strongly suggests that DOPA does not depress transmission generally in the cord but acts selectively on some pathways

In many ways the effect of DOPA resembles the tonic inhibition of transmission that exists in the decerebrate preparation In this state there is also a profound inhibition of transmission from the FRA and Ib afferents to motoneurones (Eccles Lundberg 1959) and from the FRA to primary afferents and ascending pathways (Holmqvist, Lundberg and Oscarsson 1960 Carpenter *et al* 1963) Another similarity is that the Renshaw inhibition is increased under both conditions (cf Holmqvist and Lundberg 1959) The only difference is that component I of the DRP from cutaneous afferents, representing activity in a pathway to cutaneous afferents is never depressed in the decerebrate state (Carpenter *et al* 1963) whereas DOPA was sometimes effective in depressing this potential

Carlsson *et al* (1964 have shown that the monoaminergic terminals in the spinal cord belong to descending pathways The effect of DOPA may be due to synthesis and outflow of transmitter from a descending noradrenergic pathway which has an inhibitory effect on those spinal pathways in which transmission is depressed by DOPA. However there is another possibility Through its effect by decarboxylation outside the central nervous system DOPA may increase the catecholamine levels in the blood plasma These circulating catecholamines presumably do not enter t

spinal cord because of the blood brain barrier but could cause vasoconstriction and ischaemia in the spinal cord. It is possible that the effect of i.v. or i.a. injected NA on the spinal reflex transmission (cf. McLennan 1963) is caused mainly by ischemia. That DOPA does not act in this way is strongly suggested by the selectiveness of its effect, by the fact that DA (that does not enter the spinal cord but acts and gives NA formation in the periphery) in doses which give the same blood pressure rise as DOPA has no effect and by the fact that the effect of DOPA at any stage can be reversed within a few minutes by the adrenergic α blocker phenoxybenzamine, while a comparable anoxic depression of transmission from the FRA only is very slowly reversed after admittance of oxygen (unpublished observations). Results supporting the working hypothesis that DOPA acts by liberating transmitter from a descending NA pathway are presented in a separate paper (Andén, Jukes and Lundberg 1966).

The similarity between the effect of DOPA and the tonic decerebrate inhibition of transmission in spinal reflex pathways and to ascending pathways raises the question whether the latter action is maintained by a descending noradrenergic pathway. However, this possibility was not supported by experiments in which phenoxybenzamine gave no release of spinal reflex transmission from the tonic decerebrate control (unpublished observations).

Our finding that DOPA depresses transmission from the FRA was unexpected in view of the fact that this drug increases the flexor reflex evoked by pinching of the skin in the acute spinal cat (Carlsson *et al.* 1963). DOPA often depresses transmission to primary afferents more effectively than transmission to motoneurones and it was therefore necessary to investigate whether or not DOPA increases the flexor reflex by removing presynaptic inhibition. As already discussed above our results show that this hypothesis can be discarded. There is no doubt that the reason for the increased flexor reflex after DOPA has been given is the late long lasting discharge that is evoked in flexor motoneurones from the FRA. The long latency is not caused by a peripheral loop but represents a long central delay. This pathway, as well as the pathway giving a late DRP in primary afferents after DOPA, has been analysed in detail as will be reported separately (cf. preliminary reports Andén, Jukes *et al.* 1964, Jankowska *et al.* 1965). It is postulated by these authors that the primary effect of DOPA is to depress transmission in the short latency paths from the FRA and that the late actions from the FRA are released when the former pathways are inhibited by DOPA.

One more question remains to be discussed. The inhibitory effect of DOPA on transmission may be produced either by inhibition at an interneuronal level or by presynaptic inhibition caused by a PAD. Indirect evidence suggests the former mechanism. First there is the fact that the transmission from Ib afferents to motoneurones is depressed whereas transmission from these afferents to primary afferents is uneffected by DOPA. Since it is unlikely that there are differential effects on terminals from the same afferents within the same region in the spinal cord this strongly suggests that DOPA inhibits transmission to motoneurones from Ib afferents at an interneuronal level. Second, it should be noted that while the early pathways

activated from the FRA are very effectively inhibited there are late effects evoked from the FRA after DOPA The latter actions are much easier to understand if it is assumed that DOPA inhibits transmission in the short latency path from the FRA at an interneuronal level (cf Lundberg 1966)

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The Effect of DOPA on the Spinal Cord.

2. A Pharmacological Analysis

By

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Abstract

Andén, N.-E., M. G. M. Jukes and A. Lundberg. *The effect of DOPA on the spinal cord. 2. A pharmacological analysis.* Acta physiol. scand. 1966. 67. 387—397.

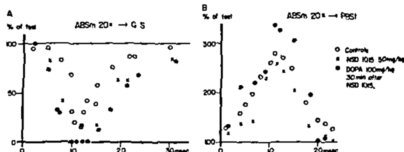
Drugs known to influence noradrenergic transmission were investigated with respect to their action on the effect of DOPA on transmission in the spinal cord. The effect of DOPA may be prevented by inhibition of DOPA decarboxylase and by pretreatment with reserpine; it is enhanced by inhibition of monoamine oxidase and reversed by the adrenergic α receptor blockers phenoxybenzam[illegible]

An i.v. injection of DOPA (l-3,4-dihydroxyphenylalanine) profoundly changes neuronal transmission in the spinal cord. The main effect is that transmission of short latency effects from the FRA (flexor reflex afferents) is inhibited and that late, long lasting actions, which are not normally found in the acute spinal cat, are evoked from these afferents (Andén *et al.* 1966). DOPA is an intermediate in the biosynthesis of catecholamines (dopamine, DA; noradrenaline, NA; adrenaline, A) and Carlsson *et al.* (1964) have demonstrated noradrenergic nerve terminals in the spinal cord, which belong to descending spinal pathways. On this basis Andén *et al.* (1966) put forward the working hypothesis that DOPA acts on transmission from the FRA by giving increased synthesis and release of transmitter from the terminals of a descending noradrenergic pathway, which inhibits transmission in some paths from the FRA. This working hypothesis has been tested in the present investigation in which the effect of DOPA has been studied after administration of drugs known to affect noradrenergic transmission. Some results on chronic spinal cats will also be reported. A preliminary report has been published (Andén, Jukes and Lundberg 1964).

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TABLE I. Levels of noradrenaline and dopamine in cat lumbar enlargement after different treatments. The figures are single values or means with the range in brackets

Treatment	Noradrenaline (μg/g)	Dopamine (μg/g)
No pretreatment (n = 6)	0.23 (0.13–0.34)	0.0
L-DOPA (67—100 mg/kg, 1 hr) (n = 5)	0.34 (0.23–0.42)	3.5 (2.6–4.7)
NSD 1015 (50 mg/kg, 1½ hr) + L-DOPA (100 mg/kg, 1 hr)	0.24	0.1
Reserpine (2 mg/kg, 7 hrs) + L-DOPA (67 mg/kg, 1 hr)	0.03	3.2
Reserpine (3 mg/kg, 17 hrs + 2 mg/kg, 2 hrs) + L-DOPA (100 mg/kg, 1 hr)	0.00	2.3
Reserpine (3 mg/kg, 17 hrs + 2 mg/kg, 3 hrs) + L-DOPA (100 mg/kg, 2 hrs + 100 mg/kg, 1 hr)	0.00	1.7



[illegible] cord the ordinate expresses the percentage increase or decrease of the conditioned response compared to the unconditioned test (100 %). The small increase in inhibition shown in A after the administration of the decarboxylase inhibitor NSD 1015 (50 mg/kg) is not significant. DOPA (100 mg/kg) was injected 30 min after NSD 1015 and had no effect.

Methods

[illegible]

Results

I. *Decarboxylase inhibition*

The first step in the synthesis of NA from DOPA is the decarboxylation giving DA. In 3 expts. the effect of DOPA was investigated after administration of the DOPA decarboxylase inhibitor NSD 1015 (*m*-hydroxybenzylhydrazine) (Carlsson 1964) and practically no formation of DA could be found in these animals (Table I).

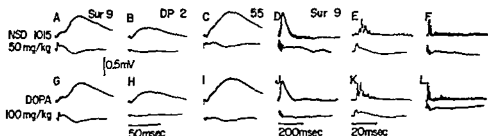


Fig 2 Blockade of the effect of DOPA on DRPs and ventral root discharges by pretreatment with an inhibitor of DOPA decarboxylase The upper traces in A D and G-J are DRPs in E F K and L ventral root discharges evoked by stimulation of nerves at the strengths indicated (multiples of threshold), the lower traces are recorded at the dorsal root entry zone Records A—F were taken 30 min after the injection of the decarboxylase inhibitor NSD 1015 (50 mg/kg) which produced [illegible]

This drug has no significant effect on transmission from the FRA, the slight increase of the inhibitory effect from high threshold muscle afferents in curve A, Fig 1, is almost certainly not significant DOPA given 30 min after NSD 1015 did not change the effect from high threshold muscle afferents on extensor and flexor test reflexes (Fig 1 A, B) Without NSD 1015 this dose of DOPA would have very effectively decreased the conditioning effects (Anden *et al* 1966) Fig 2 shows that when DOPA was given after the decarboxylase inhibitor it depressed neither the discharges in the ventral roots nor the DRPs evoked from the FRA and that no late DRP or ventral root discharge was evoked from the FRA It is therefore postulated that DOPA must be decarboxylated to exert its effect on transmission from the FRA

DOPA is a precursor of the catecholamines DA, NA and A but the effect on transmission from the FRA must be exerted either through DA or NA because A is not produced in detectable amounts from DOPA in the spinal cord

II *DOPA after reserpine*

An i v injection of DOPA increases the concentration of DA and NA in the CNS However, after pretreatment with reserpine DOPA does not increase the NA level in rabbit brain tissue whereas DA increases to about the same level as in untreated animals (Carlsson 1959)

Information as to whether DOPA acts on transmission from the FRA by producing DA or NA has been obtained from experiments in which DOPA was given after reserpine Administration of reserpine itself has no effect on transmission from the FRA if no monoamine oxidase (MAO) inhibitor has been given previously (cf Andén *et al* 1964) Reserpine was given to cats about 20 hrs before the acute experiments Fig 3 illustrates that under these conditions DOPA had very little effect There is a slight decrease of the DRP in G and H after 100 mg/kg I there was no significant depression of the ventral root discharge (I) it

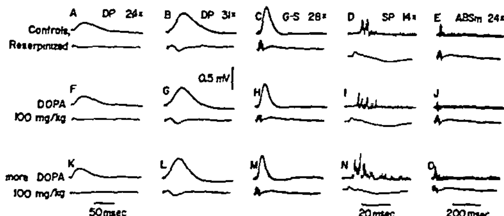


Fig 3 Blockade of the effect of DOPA by pretreatment with reserpine Upper traces, records A—C, F—H and K—M, show DRPs and D, E I, J N and O ventral root discharges, evoked by stimulation of nerves at the strengths indicated (multiples of threshold), the lower traces of all [illegible]

DRP

any late effects produced in dorsal and ventral roots (H and J) Furthermore an additional dose of DOPA had no effect This finding is in striking contrast to the effect of DOPA without treatment with reserpine (Anden *et al* 1966) Analyses of the DA and NA concentrations in the spinal cord reveal that when DOPA is given after reserpine DA increased to almost the same levels as in non-reserpinized cats but that there was no increase in the NA concentration (Table I) On this basis it is suggested that DOPA normally acts largely through synthesis of NA and only to a minor extent through synthesis of DA

Another possibility was the use of dl threo-3,4-dihydroxyphenylserine DOPS) which is decarboxylated to NA directly and not via DA (Schmiterlow 1951, Carlsson 1964) dl-DOPS in doses up to 400 mg kg was given in 3 expts but no effect was observed on transmission from the FRA even after pretreatment with nialamide (50 mg kg) and α-propyldopacetamide (100 mg kg) (Carlsson Corrodi and Waldeck 1963) to inhibit the MAO and the catechol-O-methyltransferase respectively The probable explanation is that DOPS is decarboxylated only very slowly in the cat spinal cord, there was only a very slight increase in the NA level in the spinal cord in these experiments

III *MAO inhibition*

NA may be inactivated through MAO and probably also through the catechol-O-methyltransferase We have investigated the effect of DOPA after administration of

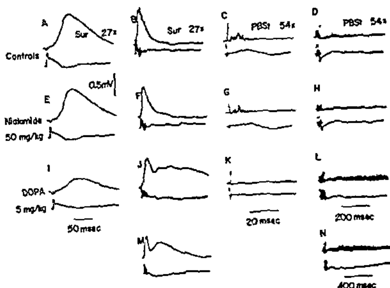


Fig 4 The potentiation of the effect of DOPA by a monoamine oxidase inhibitor The upper

the MAO inhibitor nialamide (50 mg/kg i v) In Fig 4 nialamide has not produced any significant effect after 30 min After i v injection of 5 mg/kg of DOPA there was a pronounced depression of transmission from the FRA as is illustrated for effects evoked from cutaneous afferents in I and high threshold muscle afferents in K Furthermore the late effects were evoked from the FRA in primary afferents (J) and in motoneurones (L) Without nialamide this dose of DOPA is ineffective and to produce the effects in I—J it is usually necessary to give between 50 and 100 mg/kg DOPA Hence nialamide produced at least a tenfold potentiation of the effect of DOPA

We have previously reported that some time after administration of nialamide there may be a depression of transmission from the FRA Anden *et al* 1964) Further experiments have shown that when this occurs it is the exception rather than the rule In several experiments there was no depression of transmission from the FRA during 4 hrs following a dose of 50 mg/kg nialamide This suggests that spontaneous liberation of monoamines usually is not large enough to give effect of transmission from the FRA

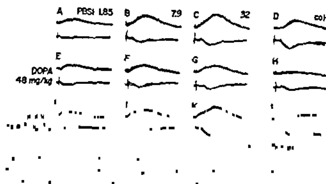


Fig 5 Partial reversal of the effect of DOPA by chlorpromazine Upper traces of each pair are the DRPs evoked by stimulation of nerves at the strengths indicated (multiples of threshold), the lower traces are the potentials recorded from the

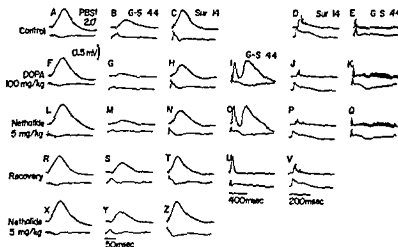


Fig 6 The inability of a β-receptor blocker to reverse the actions evoked from the FRA after DOPA

evoked from the group I afferents (X) and high threshold muscle and skin afferents ... be seen The time scale for the first three left hand columns is that shown under Y the time scale in U also applies to the right hand column The voltage calibration refers to the DRP

IV. *Receptor blockers*

Andén *et al* (1966) demonstrated that the effects of DOPA are completely reversed by phenoxybenzamine (20 mg/kg i v) Fig 5 shows that chlorpromazine also has some effect There is a partial reversal of the inhibitory effect of DOPA on transmission to primary afferents The blocker of the adrenergic β-receptor nethalide (5–10 mg/kg i v) was tried in 3 expts In the experiment illustrated by Fig 6 it was given after DOPA and there was a slight increase of the early DRPs evoked from the FRA (L—N) but no effect on the ventral root discharge nor on the late

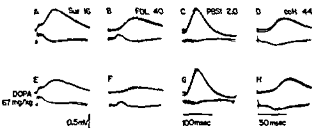


Fig 7 The effect of DOPA in a chronic spinal cat The experiment was made on a cat in which 4 weeks previously the spinal cord had been transected in the lower thoracic region The upper traces are DRPs and the lower traces are the potentials recorded from the L7 dorsal root entry zone A—D were taken before and E—H 5 min after intravenous injection of DOPA. Time calibration below H refers to all records except C and G

TABLE II Effects of L-DOPA injection on the levels of noradrenaline and dopamine in cat spinal cord cranial and caudal to a chronic or acute transection in the lower thoracic region

		Noradrenaline (μg/g)		Dopamine (μg/g)	
Days after transection	Treatment	Cranial to transection	Caudal to transection	Cranial to transection	Caudal to transection
13	L-DOPA 67 mg/kg, 1 hr	0 13	0 03	1 5	1 1
20	L-DOPA 33 mg/kg, 1 hr	0 08	0 02	1 7	1 0
21	L-DOPA 67 mg/kg, 1 hr	0 09	0 01	1 2	0 3
28	L-DOPA 67 mg/kg, 1 hr	0 16	0 01	2 0	0 6
0	L-DOPA 80 mg/kg, 1 hr	0 16	0 31	2 6	3 1
0	L-DOPA 67 mg/kg, 1 hr	0 10	0 24	1 4	3 3
0	L-DOPA 67 mg/kg 1 hr	0 15	0 26	1 7	2 3
0	L-DOPA 67 mg/kg, 1 hr	0 09	0 21	2 0	2 5

actions evoked from the FRA (O and Q) However, nethalide also increased the DRP evoked by group I volleys from PBSt (L), this suggests that the effect in M and N was not caused by a blockage of the DOPA action but by the ability of nethalide to increase the DRP Further evidence that this was so was obtained when nethalide was given late in the experiment of Fig 6 In R—V there is recovery from DOPA and when nethalide was given again it again increased the DRPs (X—Z) In conclusion the β-receptor blocker nethalide, although having certain effect on DRPs, does not block the action of DOPA The effect by nethalide on the DRPs resemble that produced by anaesthetics (Eccles, Schmidt and Willis 1962) However, it is possible that the increase in the DRPs in the two cases are produced by different mechanisms because anaesthetics in low doses very effectively depress transmission of the late effects to primary afferents and motoneurones (Jankowska, Jukes, Lund and Lundberg, unpublished), while nethalide had no effect on the late potential (Fig 6)

V *Chronic spinal cats*

If DOPA acts via a descending noradrenergic pathway it is of interest to examine the effect in chronic spinal animals. This was done in 4 expts. in which the spinal cord had been transected 2—4 weeks before the acute experiments. In all experiments DOPA depressed transmission from the FRA. The effect was weaker than in the acute spinal animal and the action in Fig. 7 was the most marked found in this series. A total dose of 67 mg/kg was given to each of these cats. In 3 of the experiments we first examined the effect of half of this dose and in all cases a maximal effect was produced by this first dose and there was no increase when the remaining 33 mg/kg DOPA were given. This suggests that the effect of DOPA is decreased in the chronic spinal cat but that a maximal effect is achieved by a smaller dose of DOPA. The explanation may be supersensitivity caused by denervation in combination with a decrease in the amount of enzymes converting DOPA to catecholamines (see Table II). However, in order to prove this hypothesis a much more extensive investigation should be performed. First it is necessary to examine the effect of large doses of DOPA, second it is necessary to prove that also in chronic spinal cats DOPA only acts after decarboxylation, i.e. that the supersensitivity of denervation has not made the cells sensitive to DOPA.

Discussion

Drugs known to influence the synthesis, liberation, inactivation and receptor attachment of NA have been investigated with respect to their action on the effect of DOPA on transmission in the spinal cord. The purpose has been to test the working hypothesis that DOPA acts on transmission from the FRA by liberating transmitter from a descending noradrenergic pathway (cf. Andén *et al.* 1966).

Since DOPA has very little or no effect after inhibition of the decarboxylase (Fig. 1) and its effect is potentiated after MAO inhibition (Fig. 4) it is concluded that it acts only after decarboxylation and not directly. This is of interest since DOPA is an amino acid and many of them have strong effects on central neurones (Curtis and Watkins 1960). Decarboxylation of DOPA gives DA which through the action of dopamine β hydroxylase is converted to NA. DOPA could act on transmission from the FRA through the formation of DA or NA or both of these substances. Most of the DOPA decarboxylase in the spinal cord is in descending fibres (Andén, Magnusson and Rosengren 1965) but the capillary wall contains some decarboxylase (Bertler, Falck and Rosengren 1963). The possibility must therefore be considered that DOPA may act through DA produced when DOPA passes through the capillary wall. In this connection it seems relevant that DOPA has little or no effect after pretreatment with reserpine. Reserpine is known to affect monoaminergic nerve terminals but there is no reason to believe that reserpine should have effect on the decarboxylase in the capillary wall. It is therefore unlikely that the action of DOPA in the acute spinal cat is caused by DA formation in the capillary walls and it is suggested that the effect is exerted after decarboxylation in descending terminals. We have no direct evidence showing whether the effect of DOPA is

produced by DA or NA but the finding that DOPA has little effect after pretreatment with reserpine is probably significant also in relation to this problem The biochemical analyses show that after reserpine DOPA does not produce an increased level of NA in the spinal cord whereas the DA concentration rises to almost the same level as without reserpine It is therefore suggested that DOPA acts in the acute spinal cat largely through liberation of NA In this connection it is of interest that amphetamine has similar effects as DOPA on spinal cord transmission (Vyklicky and Tabin 1964)

NA is formed from DA through the action of dopamine β hydroxylase which we assume exists only in NA neurones Further tests with inhibitors of this enzyme would be valuable If DOPA to some extent may act through DA in the acute spinal cat the decarboxylation is likely to occur in terminals of a descending noradrenergic pathway The terminals of descending 5 hydroxytryptaminergic pathways in all likelihood also contain the same decarboxylase but DOPA is probably not taken up in these terminals to any large extent since 5 HT neurones usually do not display catecholamine fluorescence when DOPA is given after MAO inhibition (Fuxe 1965) The relationship between the effects evoked by DOPA and 5 HTP will be discussed in a forthcoming paper (cf preliminary report Andén *et al* 1964)

When the present experiments were made there was no evidence that spinal neurones were influenced by electrophoretically applied NA (Curtis Phillis and Watkins 1961) Subsequently Engberg and Ryall (1965 1966) have shown that many interneurones in the spinal cord are inhibited by NA but in no case was there evidence of excitatory effect Engberg and Ryall (1965) suggest that NA acts as an inhibitory transmitter in the spinal cord and that the noradrenergic descending pathway, through which DOPA is assumed to act has direct connection with the interneurones transmitting short latency effects from the FRA The effect of DOPA can be completely reversed by phenoxybenzamine (Anden *et al* 1966) and partially reversed by chlorpromazine (Fig 5) but not by nethalide (Fig 6) Since the former substances act as blockers of adrenergic α receptors it was suggested that NA produced from DOPA acts on α receptors (Anden *et al* 1963 Anden *et al* 1964 Lundberg 1965) However Engberg and Ryall 1966) have shown that the effect on interneurones of electrophoretically applied NA cannot be blocked by phenoxybenzamine or nethalide and suggest that NA acts in the spinal cord on receptors that are neither of α nor β type Tentatively they have suggested that phenoxybenzamine blocks the effect of DOPA in the presynaptic NA nerve terminals Further experiments are required to elucidate this problem which is important for the further analysis of the effect of DOPA cf below

Histochemical experiments Carlsson *et al* 1964 Dahlstrom and Fuxe 1965) in conjunction with various biochemical investigations of catecholamines and enzymes acting upon them for reference see Anden *et al* 1966 have virtually proved that there are descending noradrenergic pathways in the spinal cord Taken together the present results suggest that DOPA acts via noradrenergic nerve terminals and hence that there i a descending NA pathway which influences transmission

the FRA Any alternative hypothesis requires the assumption that there are neurones with NA receptors which are not utilized in noradrenergic transmission and that the NA acting upon them either is liberated from noradrenergic terminals on other cells or else that the enzymes for synthesis of NA from DOPA exist outside noradrenergic nerve terminals However, the existence of such a pathway can hardly be proved by experiments based only on the effect of DOPA It is either necessary to stimulate the pathway (which is difficult because there are several descending pathways with similar effects on transmission from the FRA (*cf* Lundberg 1966)), or else to liberate NA in some other way than by giving DOPA, for example with injections of reserpine after inhibition of the MAO (Anden *et al* 1964) Either approach requires access to a blocker known for certain to act on the postsynaptic receptors

Another obvious way of analysing the effect of DOPA was to investigate its action in chronic spinal cats Two to 4 weeks after transection of the spinal cord DOPA has similar, although weaker, effects as in the acute spinal cat These results do not falsify the hypothesis that DOPA normally acts by liberating transmitter from a descending noradrenergic pathway Even 2—4 weeks after transection of the descending pathway, enzymes remaining in the degenerating nerve terminals may produce NA from DOPA, and effects might be produced by small amounts of NA because of denervation supersensitivity of the receptive cells However, the biochemical analysis did not reveal any formation of NA from DOPA in the spinal cord below the transection in these cats and another perhaps more likely explanation is that while normally DA at the most has a weak action it may be effective during denervation supersensitivity DA could be produced either by the enzymes in degenerating terminals or during the passage of DOPA through the capillary walls (cf Bertler, *et al* 1963, Anden *et al* 1965) Longer degeneration times are required and experiments are in progress on adult cats that were spinalized at birth

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Reciprocal Effects on α- and γ-Motoneurones of Drugs Influencing Monoaminergic and Cholinergic Transmission

By

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Abstract

ARVIDSSON, J, B-E ROOS and G STEG *Reciprocal effects on α- and γ-motoneurones of drugs influencing monoaminergic and cholinergic transmission* Acta physiol scand 1966 67 398—404

The reflex responses of α and γ-motoneurones to dorsal root stimulation were recorded in fine [illegible] administration of drugs facilitating MA transmission (L-DOPA) or inhibiting ACh transmission (atropine) α motoneurone activation decreased and γ-motoneurone activation increased The drugs had always reciprocal effects on α- and γ-discharges i e inhibition of the one type accompanied facilitation of the other A hypothetical model is presented for the interpretation The reciprocal [illegible] ACh [illegible] ACh [illegible] muscle [illegible] es by [illegible]

The present work originates from the finding that reserpine, which inhibits monoamine (MA) transmission, facilitated α-motoneurones while it inhibited γ-motoneurones (Steg 1964) A subsequent injection of L-DOPA restored MA transmission and reversed the changes of α- and γ-motoneurone excitability Roos and Steg 1964) Reserpine induced rigidity, L-DOPA abolished it

This work shows the influence on α- and γ-motoneurone reflex responses of physostigmine, which facilitates, and atropine, which inhibits acetylcholine (ACh) transmission The changes of α- and γ-motoneurone excitability are quantified and a comparison is made between the effects of drugs influencing MA transmission and those influencing ACh transmission

Central MA transmission is influenced in different ways by the drugs used in this study Reserpine depletes the granules of the presynaptic terminal of MA transmitters Haloperidol and phenoxybenzamine block the postsynaptic receptors for MA transmitters In either case inhibition of MA synaptic transmission occurs If L DOPA (precursor of the catecholamines) is administered to a reserpinized animal the catecholamine transmitter is again made available and transmission is restored (Carlsson *et al* 1957, Carlsson 1959) Physostigmine facilitates ACh transmission by blocking cholinesterase Atropine inhibits ACh transmission by blocking postsynaptic receptors for the transmitter

The drug actions changing the relation between the α and γ motoneurone excitabilities have implications for motor control The α motoneurones innervate extrafusal muscle fibres, the γ motoneurones innervate the intrafusal muscle fibres of the muscle spindles The muscle spindles are length receptors According to the servo theory of muscle control the γ motor axons set the length of the intrafusal fibres and the contraction of extrafusal muscle is regulated by the stretch reflex to a length corresponding to that of the intrafusal fibres Both maintenance of posture and initiation of movement are assumed to be dependent on this "length servo" At high α and low γ motoneurone activity the equilibrium between extrafusal and intrafusal *contraction is changed into extrafusal dominance, the muscle spindle* sensitivity is lowered and a deficiency of proprioceptive information and length regulation follows At low α- and high γ motoneurone activity on the other hand there is a dominance of intrafusal contraction, increased muscle spindle sensitivity and increased proprioceptive inflow through the afferent limb of the stretch reflex arc Motor control is exerted predominantly over the direct α route in the former case, and predominantly over the indirect γ route in the latter (For review on muscle spindles and motor control see Matthews 1964)

Methods

The study is based on experiments in 18 rats The animals were operated in fluothane anesthesia After recovery from fluothane anesthesia when the operation was finished the animals were kept in a superficial Viadril[R] anesthesia

The rats were laminectomized In the earlier experiments the recording electrodes were attached to the thin intact ventral root of the third coccygeal segment and the stimulating electrodes to the corresponding dorsal root Later reflex responses to dorsal root stimulation were recorded in ventral root filaments at the L6 level Two pairs of recording electrodes 6—10 mm apart were used [illegible]

spikes *are of high* amplitude and sharp *the γ-potentials have a low amplitude and rounded form*

The number of individual α and γ potentials appearing in a reflex were counted The number of γ-potentials were plotted on the abscissa and the number of α potentials on the ordinate Fig 1) [illegible]

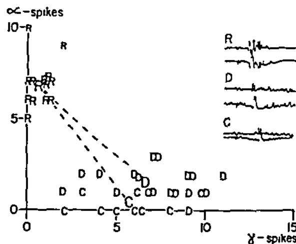


Fig 1 Reflex responses of α and γ motoneurones recorded in a thin coccygeal ventral root on dorsal root stimulation α potentials have high amplitude and short conduction time between the two pairs of recording electrodes γ potentials are identified by low amplitude and long conduction time between the two sweeps Reflex tests were made before drug administration (C) and after reserpine (R) and L-DOPA (D) injection The number of γ-potentials is plotted on the abscissa and the number of α potentials on the ordinate The mean values are indicated by large symbols Reserpine treatment raised the number of α potentials and lowered the number of γ potentials in the reflex response L-DOPA reversed the changes The deviations from the mean values in each test group were small compared to the drug induced changes

When an increase of the α reflex and a decrease of the γ reflex response is produced by for instance reserpine the α/γ-quotient increases The drug induced change of the quotient is expressed by the factor F in the equation $\frac{\alpha_1}{\gamma_1} = F\,\frac{\alpha_0}{\gamma_0}$ where α_0 and γ_0 are control values and α_1 and γ_1 are test values after drug administration $F = \frac{\gamma_0}{\alpha_0}\,\frac{\alpha_1}{\gamma_1}$ Since α_0 and γ_0 are constant and the results show that $_1$ varies approximately in proportion to $\frac{1}{\alpha_1}$ the function is exponential The drug induced change in the relation between α and γ motoneurone responses expressed as F is then plotted on a logarithmic scale (ordinate) against time (abscissa) (diagram B in Fig 2—5) It should be considered that α or γ values approaching 0 strongly influence F

Continuous lines in the diagrams indicate experiments on the L 6 segmental level interrupted lines experiments on the coccygeal level

Results

Fig 1 demonstrates the numbers of α and γ-potentials in the individual reflex tests before drug administration (C) and after reserpine (R) and L DOPA (D) treatment The deviations from the mean value in each test group are considerably smaller than the drug induced changes

In the earlier experiments at the coccygeal level the stimulation intensity varied between 1 1 and 2 times the threshold for low threshold afferents (T) The stimulation intensity was 1 5 T in the experiments on the L6 level The influence of stimulation intensity is demonstrated in Fig 2 The slope of the curves indicating the drug induced change in α and γ responses is depending of stimulus intensity (Fig 2 A) At stimulation intensity 1 1 T the slope expressed as the quotient $\frac{\alpha_1-\alpha_0}{\gamma_1-\gamma_0}$ was

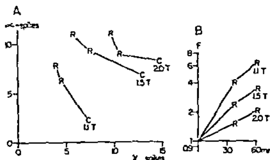


Fig 2 Effect of reserpine on the number of α and γ-potentials responding to dorsal root stimulation at 1 1, 1 5 and 2 times threshold for low threshold afferents L6 segment At weak (1 1 T) stimulation the drug induced increase of α motoneurone responses is higher than at strong (2 T) stimulation (A) F expressing the drug induced change in the relation between α and γ-responses (see text) is higher at weak than strong stimulation (B)

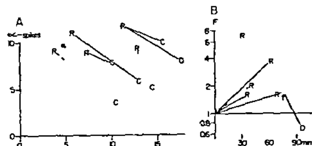


Fig 3 The reciprocal effects of 6—7 mg/kg reserpine (R) and 100 mg/kg L-DOPA (D) on α and γ-motoneurone reflex responses in 3 lumbar (continuous lines) and 2 coccygeal (interrupted lines) experiments C represents control tests before drug action A) For each point the ordinate represents the mean number of α potentials in 5—10 reflex exposures and the abscissa the mean number of γ-potentials in the same reflex tests (see Fig 1) B) The drug induced change in the relation between α and γ-motoneurone responses F (see text) is plotted on the ordinate against time on the abscissa F is raised by reserpine and lowered by L-DOPA Arrow indicates the time of L-DOPA injection

—1 7 At stimulation intensity 2 T, the slope was —0 56 Independently of stimulus intensity the sign was negative, that is α increase was accompanied by γ-decrease

The F factor also demonstrates the influence of stimulation intensity (Fig 2 B) At low stimulus intensity F was high F was lowered by increasing stimulus intensity Drug treatment raised the α response 3 4 times at 1 1 T stimulation and 1 3 times at 2 T The corresponding decrease of the γ response at 1 1 T was 1 8 and at 2 T it was 1 5 Obviously the greater part of the change of F was due to a change of the α response The drug induced increase of the α response to dorsal root stimulation is low when a strong stimulus recruites a large fraction of the α motoneurones of the pool The increase of the α response is great when only a small fraction of the pool is recruited by a weak stimulus

The diagrams of Fig 3 demonstrated quantitatively the effect of reserpine and L-DOPA on α and γ motoneurone excitability Reserpine induced similar shifts to higher α and lower γ values in all experiments L DOPA reversed the changes (see also Fig 5) Reserpine raised F the factor expressing the changed relation between α and γ motoneurone excitabilities L-DOPA lowered F to values below 1

Fig 4 demonstrates the effects on α and γ motoneurones when first physostigmine and then atropine were injected Physostigmine administration induced a change to higher α and lower γ-excitability values Atropine reversed the physostigmine induced changes F was raised by physostigmine and lowered by atropine,

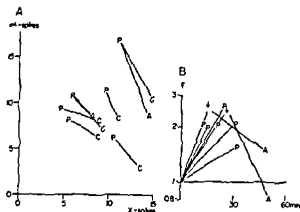


Fig 4 The reciprocal effects of physostigmine (P) and atropine (A) presented as in Fig 3 The dose of physostigmine was 0 25 mg/kg in all experiments 5 mg/kg of atropine partially reverses the physostigmine effect After 8 mg/kg the factor F was lowered below the initial value of 1

excitabilities into γ-dominance

by a high dose of atropine, even below 1 Like reserpine physostigmine induced rigidity. Atropine abolished physostigmine rigidity

When atropine was given as the first drug (Fig 5) the α-excitability decreased, the γ-excitability increased and the F values became lower than 1 Later injections of reserpine, phenoxybenzamine or haloperidol reversed the atropine changes and F increased above 1 The experiments of Fig 5 were made early when the stimulation intensity had not yet been standardized at 1 5 T but varied between 1 1 and 2 T The greater variations in the slopes of the lines indicating the drug effects in Fig 5 compared with Fig 3 and 4 might be attributed to the variations in stimulus intensity.

Discussion

The drugs used in these experiments always had reciprocal effects on the reflex activation of α and γ motoneurones, i e inhibition of the one type accompanied facilitation of the other

A consequence of the reciprocal effects on α and γ motoneurones is a disequilibrium between the α and γ routes of motor control In the diagrams values of $F > 1$ imply changes in the relation between α and γ motoneurone reflex activation towards predominance for the α route of motor control — α dominance $F < 1$ indicates a change towards predominance for the γ route — γ dominance

Physostigmine, reserpine, haloperidol and phenoxybenzamine all raised the value of F and induced α dominance Atropine and L DOPA lowered the value of F and induced γ dominance

Inhibition of MA transmission (by reserpine, haloperidol or phenoxybenzamine) and facilitation of ACh transmission (by physostigmine) induced similar states of α-dominance Facilitation of MA transmission (L DOPA) had the same effect as inhibition of ACh transmission (atropine) namely γ dominance

An interpretation of these data is provided by the assumption that the reciprocal changes of α and γ motoneurone excitability are induced from antagonistic MA and ACh neurones The drug induced change in the relation between α and γ motoneurone responses to dorsal root stimulation is determined by the net effect of the antagonistic influences from the MA and ACh neurones Thus a shift of the MA ACh equilibrium in favour of the ACh neurones induced α dominance A shift in favour of the MA neurones induced γ-dominance

Although the hypothesis is only descriptive its connection to recent findings on MA and ACh neurone systems in the brain might make it useful as a basis for further neurophysiological analyses

Dopamine, which does not penetrate the blood brain barrier and thus has only peripheral actions did not influence reserpine induced rigidity or α and γ response changes This indicates a central site of reserpine action (Roos and Steg 1964) The hypothesis that the action of reserpine is supraspinal was supported by the fact that α hyperactivity and rigidity were abolished after spinal section Descending spinal MA pathways are also influenced by the drugs (Anden *et al* 1964 b) but are probably not responsible for the described effects

The mapping out of the MA neurones of the brain made possible by Hillarp s fluorescence technique (see Hillarp *et al* 1965) has demonstrated a large system of dopamine (DA) neurones with cell bodies in the substantia nigra axons passing through the crus cerebri and terminals in the neostriatum (Anden *et al* 1964 a, 1965 Bertler *et al* 1964 Anden *et al* 1966 a These DA neurones and ascending ACh neurones from the brain stem to the neostriatum (Shute and Lewis 1963) are here of special interest

In rats with chronic unilateral lesions which interrupted the nigro-neostriatal DA neurones reserpine and L DOPA induced asymmetric postures (Anden *et al*

1966 b) and unilateral reciprocal changes of the α and γ motoneurone excitabilities (Anden, Larsson and Steg 1966) The same reciprocal drug actions as described above are found on the intact side but not on the side of the lesion

The analysis is carried further on the working hypothesis that the neostriatum exerts reciprocal influences on the activity of α and γ motoneurones and that the net effect of the antagonistic influences from the DA and ACh neurone systems directs this reciprocal control

This work was supported by grants from the Swedish Medical Research Council (Project No 14X 165-02 and B67 21X 622 02)

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Acta physiol scand 1966 67 405—410

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Monoamines in Brain and Adrenal Glands of Cats after Electrically Induced Defense Reaction

By

LARS M GUNNE and TOMMY LEWANDER

Abstract

GUNNE LARS M and TOMMY LEWANDER *Monoamines in brain and adrenal glands of cats after electrically induced defense reaction* Acta physiol scand 1966 67 405—410

The defense reaction ("sham rage) was produced in cats by electrical stimulation of the amygdaloid nucleus or [illegible]
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A lowering of the brain noradrenaline (NA) content has been noted in cats after electrical stimulation of certain brain areas notably the amygdaloid nucleus (Gunne and Reis 1963) The effect on the brain catecholamines (CA) was found to be closely related to the behavioral manifestations of rage (Reis and Gunne 1965) By histochemical technique a depletion of the noradrenergic terminals was shown to occur within practically all regions of the forebrain both ipsi and contralateral to the site of stimulation (Fuxe and Gunne 1964)

The present study demonstrates the effects on brain and adrenal gland CA and brain 5 hydroxytryptamine (5HT of stimulation of the amygdaloid nucleus in cats pretreated with α propyldopacetamide H22 54 a potent inhibitor of the amine synthesis In a few amygdaloid stimulated cats a complete chromatographic separation of the brain amines was undertaken in order to measure not only the brain CA but also the corresponding methoxylated amines normetanephrine (NM and methoxytyramine (MT) Furthermore a study was made of the effects on brain and adrenal gland amines of electrical stimulation of a different brain region the lateral hypothalamus (median forebrain bundle A preliminary report of these data has been give [illegible]

AMYGDALA STIMULATION HYPOTHALAMUS STIMULATION

Fig 1 Fig 2

Fig 1 Monoamine content in per cent of control values (dotted line) of cat brains and adrenal glands after electrical stimulation of the amygdaloid nucleus producing defense reaction Vertical bars standard error of the mean NA-noradrenaline, DA dopamine A adrenaline 5HT- 5-hydroxytryptamine

Fig 2 Monoamine content in per cent of control values (dotted line) of cat brains and adrenal glands after electrical stimulation of the lateral hypothalamus. Symbols see text to Fig 1

Methods

[illegible]

with sedation or sleep (n = 4) 6) amygdaloid stimulated animals pretreated with H 22/54 200 mg/kg i p 15 min before the onset of stimulation (n = 4) and 7) animals stimulated within the lateral hypothalamus (n = 6)

The physiological and chemical methods are described in detail elsewhere (Gunne and Reis 1963, Gunne 1963) Under nembutal anesthesia the animals had single stainless steel Hess electrodes implanted into the right basomedial amygdaloid nucleus (stereotaxic coordinates according to Jasper and Ajmone Marsan (1954) AP = 11 0 L = 10 0 H = − 4 0) or the right lateral hypothalamic region (AP = 11 5 L = 3 0 H = − 3 0) A screw in the calvarium served as the indifferent electrode — When the animals had recovered from the after-effects of the operation usually 3 days later they were placed in observation cages (45 × 45 × 48 cm) and connected with a Grass stimulator Trains of stimulation were delivered intermittently for 3 min followed by 4 min intervals (laboratory timer according to Perman and Persson 1962) Stimulus intensity 1 10 V (depending on the behavioral response) stimulus duration 1 msec frequency (for amygdaloid stimulation) 6 c/sec (for hypothalamic stimulation) 50 c/sec The stimuli were repeated 20 25 times within 2—3 hrs and during this time the behavior and signs of autonomic activity were continuously observed

[illegible] after purification on ion exchange resins as described by Gunne (1963) A few brains were taken for a [illegible] Haggendal (1962 [illegible] whole brain extract [illegible]

photofluorimeter.

TABLE I Brain noradrenaline (NA) serotonin (5 HT) and dopamine (DA) ng/g wet tissue weight in cats The effect of rage attacks induced by electrical stimulation of selected brain areas

		Brain stem		Telencephalon	
Treatment	n	NA	5 HT	NA	DA
Controls	17	340±14	590±29	200±11	380±24
Amygdala stim (without rage)	4	310±21	650±30	190±16	340±14
Amygdala stim (rage)	7	230±22[1]	580±25	100±11[1]	470±33
Hypothalamus stim (rage)	6	180±34[1]	680±52	120±16[1]	380±45

[1] Different from controls P<0 001

Results

Electrical stimulation of amygdala and hypothalamus in most instances elicited the characteristic autonomic, somatic and behavioral manifestations of the defense reaction Some of the signs noticed were alerting, pupillary dilatation, salivation, piloerection, tachypnea, hissing, growling and attacking behavior When evoked from the amygdala the response gradually increased the attacking behavior generally not starting until the end of each period of stimulation, while the hypothalamic response was immediate in onset In four cats amygdala stimulation resulted in sedation or sleep, while attacking behavior resulted in all cats with hypothalamic electrodes Hypothalamus stimulation elicited directed attacks aimed at any moving object while amygdala stimulation caused nondirected attacks, the cats seemingly fighting into the air A group of cats pretreated with H22/54 appeared to have the same response After 1—2 hrs there were, however signs of prostration and if the stimulation was not interrupted the cats died suddenly without further premonitory symptoms

Brain and adrenal gland monoamines

The untreated controls and the operated nonstimulated controls were combined, since the amine levels of these two groups did not differ significantly

A *Amygdaloid stimulation*

Fig 1 illustrates the effects of rage attacks elicited by stimulation of the amygdaloid nucleus A decrease of NA was noted in the brain stem and telencephalon, while the brain stem content of 5HT and the telencephalon content of DA was unaltered In the adrenals a reduction of both A and NA occurred after stimulation

Electrical stimulation of the amygdaloid region without resulting rage attack did not alter the brain or adrenal gland amines Table I and II)

TABLE II Adrenal gland content of adrenaline (A) and noradrenaline (NA) µg/g wet tissue weight in cats The effect of rage attacks induced by electrical stimulation of selected brain areas

Treatment	n	Adrenals	
		A	NA
Controls	27	820±38	460±40
Amygdala stim (without rage)	5	700±98	350±36
Amygdala stim (rage)	11	320±34[1]	260±48[2]
Hypothalamus stim (rage)	*8*	*270±72*[1]	*310±88*

[1] Different from controls $P<0.001$
[2] Different from controls $P<0.01$

TABLE III Effects of amygdala stimulation (producing rage attacks) on the brain stem noradrenaline (NA) and serotonin (5 HT) ng/g and the adrenal gland content of adrenaline (A) and NA µg/g in cats pretreated with α propyl-dopacetamid (H22/54) 200 mg/kg

Treatment	n	Brain stem		Adrenals	
		NA	5 HT	A	NA
Controls	7	270±13	470±34	860±67	570±67
Amygdala stim	4	190±12	440±29	310±61	210±59

Administration of H22 54 caused a reduction of brain amines even in the controls, while the adrenal gland CA were unchanged (Table III) Amygdaloid stimulation in cats pretreated with H22/54 produced a marked decrease in brain stem NA and adrenal gland A and NA The 5HT level of the brain stem was unchanged in these cats

Separation of the amines on Amberlite CG 120 showed that the reduced NA level in the brain after amygdala stimulation was accompanied by an increase of NM, while DA and MTA were unaltered (Fig 3)

B *Hypothalamic stimulation*

Rage attacks elicited by stimulation of the lateral hypothalamic region were associated by a pattern of brain and adrenal gland amines similar to that produced by amygdala stimulation (Fig 2) A marked reduction of brain stem and telencephalic NA was seen together with a decrease of the adrenal gland content of A The brain content of DA and 5HT and the adrenal gland NA were not significantly altered

Discussion

The behavioral response to electrical brain stimulation of both the amygdaloid and the lateral hypothalamic regions generally consists of a rage attack with several

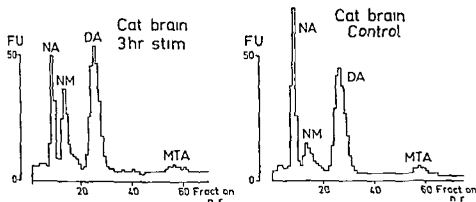


Fig 3 Chromatografic separation of the amines of single cat brains on Amberlite CG 120 Direct fluorescence of 1 3 ml fractions read at 285/335 nm (act /fluor wavelengths uncorrected) Left effect of 3 h of amygdaloid stimulation producing defense reaction. Right control The peaks are noradrenaline (NA) normetanephrine (NM) dopamine (DA) and methoxytyramine (MTA) 50 fluorescence units (F U) correspond to 1 μg NA/ml

concomitant autonomic signs In accordance with many earlier reports there were, however, great differences between the two types of rage attacks elicited from the two different brain regions (cf Delgado 1964) Amygdala stimulation caused a slowly increasing set of autonomic and somatic behaviour, ending after a few minutes of stimulation in a non directed attack when the animals seemed to be hallucinating, growling and fighting aimlessly in the air Hypothalamic stimulation, on the other hand, immediately (within a few seconds) resulted in attacks directed at any moving object, ending in a fiercly pursued outburst of aggression

Despite the differences in behavior from stimulation at these different sites, both were accompanied by similar responses of brain and adrenal gland amines Stimulation of these two brain areas seemed to activate specifically the NA containing neurons, as indicated by a decrease of brain NA and an increase of brain NM The DA containing neurons probably were not involved, since the DA level was unaltered and there was no increase of the corresponding 3 methoxylated amine MTA 5HT also remained unchanged during these stimulation experiments It seems unlikely that a liberation of 5HT would have been concealed by a simultaneous resynthesis When the resynthesis was blocked by administration of H22/54 (a potent inhibitor of both CA and 5HT synthesis) there was no reduction of 5HT compared with the non stimulated controls H22 54 in itself lowered the levels of brain amines in accordance with Corrodi (1965) The adrenal gland levels was unchanged in non stimulated animals, probably as a sign of a low turn-over of amines in the adrenals under resting conditions

The findings presented seem to indicate a close connection between a behavioral state of rage or excitation and a release of brain NA When the rage reaction failed to appear, or when the brain stimulation induced sedation or sleep no alterations of the brain amines were noticed The findings also appear to connect a some

brain NA with a stimulation of the adrenomedullary system It is hoped that studies along these lines will facilitate an understanding of mechanisms involved in states of excitation, both spontaneous and drug-induced An example of such an approach is represented by the studies of long-term effects of morphine in various animals (Gunne 1963).

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The Effect of Imipramine and Related Antidepressive Drugs on Estrus Behaviour in Ovariectomised Rats Activated by Progesterone, Reserpine or Tetrabenazine in Combination with Estrogen

by

BENGT J MEYERSON

Abstract

Meyerson, B *The effect of imipramine and related antidepressive drugs on estrus behaviour in ovariectomised rats activated by progesterone, reserpine or tetraberazine in combination with estrogen* Acta physiol scand 1966 67 411—422

The effects of antidepressive drugs were studied on estrus behaviour activated in ovariectomised rats by progesterone, tetrabenazine or reserpine in combination with estrogen On tetrabenazine-activated estrus behaviour an inhibitory effect was obtained by imipramine, desmethylimipramine, amitriptyline and nortriptyline, but not by trimeprimine All the mentioned antidepressive compounds reduced the progesterone activated heat response, however, trimeprimine and nortriptyline were effecti[illegible] shor[illegible] not [illegible] syne [illegible] the [illegible] antidepressant drugs to prevent tetrabenazine induced sedation or to induce hyperactivity (compulsive exploring behaviour) The present data further support the earlier suggestion that there exist serotoninergic pathways mediating inhibition of estrus behaviour It seems that the antidepressive compounds studied potentiate central serotoninergic mechanisms

A relationship between hormone activated estrus behaviour and central nervous monoamines in ovariectomised rats is suggested by recent investigations (Meyerson 1964 a, b, c) It was found that estrus behaviour normally brought about by treatment with estrogen in combination with progesterone (Beach 1942, Boling and Blandau 1939) was inhibited by increased central nervous monoamine levels Inhibition was especially striking after a selective increase of serotonin (Meyerson 1964, b)

It has also been shown that estrus behaviour is effectively activated in ovariectomised rats, even in the absence of progesterone treatment, by estrogen in combination with the amine depletors reserpine or tetrabenazine (Meyerson 196[illegible]

Pretreatment with imipramine or its suggested metabolite desmethyl[illegible]

(Sulser, Bickel and Brodie 1962) counteracts a number of reserpine and tetrabenazine effects in rats, such as hypothermia (Garattini *et al* 1962), palpebral ptosis (Costa, Garattini, Valzelli 1960, Sulser, Watts, Brodie 1960) and sedation (Sulser, Bickel, Brodie 1962, 1964)

It is therefore of interest to find out if imipramine, desmethylimipramine and related antidepressive agents also prevent reserpine and tetrabenazine from activating estrus behaviour The investigation is also designed to study if these antidepressive drugs interfere with the activation by progesterone

In this paper the term antidepressive drug" means only the iminodibenzyl and dibenzocycloheptane derivatives studied

Methods

Ovariectomised Sprague Dawley rats were used weighing 260—320 g The animals were housed under reversed day night rhythm (light from 9 p m to 9 a m) in air conditioned quarters maintained at 22—24° C

Groups of 10—12 rats were randomly selected Before the present investigation started all animals had been tested with 10 μg/kg estradiol benzoate followed 48 hrs later by 0 4 mg/animal progesterone The percentage of animals responding to this treatment was for all groups used in this investigation within the level of response found earlier (Meyerson 1964 a) namely 78 96 % (95 % confidence level)

$$100 - \frac{\text{Expts } \% \text{ in heat}}{\text{Controls } \% \text{ in heat}} \quad 100$$

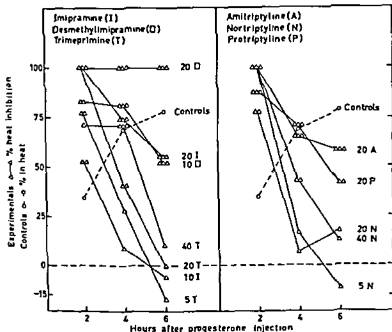
with hormones alone and in combination with tetrabenazine 40 mg/kg were run parallel as controls

Drugs injected

... throughout the investigation The doses mentioned in ...

... oil and injected in a volume of ... was diluted with distilled water ... pine sulphate pargyline HCl (Mo 911 Eutonyl® Abbott lab nialamide HCl (Niamid® Pfizer and the antidepressive drugs were dissolved or diluted in saline The antidepressive drugs were

imipramine HCl	Tofranil® Geigy
desmethylimipramine HCl	Pertofran® Geigy
trimeprimine maleate	(Surmontil® Leo
amitriptyline HCl	(Tryptizol® Merck Sharp & Dohme
nortriptyline HCl	Noritren® Lundbeck)
protriptyline HCl	(Merck Sharp & Dohme



Antidepressive drug mg/kg		Number of animals tested	Number of runs pooled	Antidepressive drug mg/kg		Number of animals tested	Number of runs pooled
Imipramine	10	35	3	Amitriptyline	20	21	2
	20	34	3	Nortriptyline	5	12	1
Desmethyl imipramine					20	11	1
	10	22	2		40	23	2
	20	12	1	Protriptyline	20	24	2
Trimeprimine	5	12	1				
	20	22	2				
	40	10	1	None (controls)		32	3

The number of triangles (△—△△△) at the various points of the graph indicate the degree of statistical significance of the inhibitory effect not the number of observations

P	>0 05	<0 05 0 01	<0 01
Symbol	△	△△	△△△

Fig 1 The effect of different antidepressive drugs on estrus behaviour in ovariectomised rats injected with estradiol benzoate 10 μg/kg at zero hrs followed 50 hrs later by 0 4 mg animal progesterone The antidepressive drugs were administered at 48 hrs

TABLE 1 The effect of imipramine on estrus behaviour activated in ovariectomised rats by estradiol in combination with reserpine and/or progesterone

Estradiol benzoate, 10 µg/kg was injected at zero hours

Treatment		Results						
			Animals in beat				Number of	
				Experimentals				
At 48 hrs	At 50 hrs	Hours after progesterone or reserpine injection	Controls %	%	% inhibition	P for difference between expts and controls	animals tested	runs pooled
A Imipramine 20 mg/kg	Reserpine, 1 mg/kg (controls had reserpine alone)	2	10	0	100	< 0.05		
		4	71	14	80	< 0.01	20–21	2
		6	83	33	60	< 0.01		
B *Imipramine,* 20 mg/kg	*Progesterone, 0.4 mg/* animal (controls had progesterone alone)	2	34	0	100	< 0.01		
		4	69	18	74	< 0.01	32–34	3
		6	78	35	55	< 0.01		
C Imipramine 20 mg/kg	Progesterone 0.4 mg/ animal and reserpine 1 mg/kg	2		5				
		4		52		0.01[1]	21	2
		6		71		< 0.05[1]		

[1] The value of P concerns the difference between treatment C and A
Treatment B values taken from fig 2

Results

Progesterone activated estrus behaviour and antidepressive drugs

Estrus behaviour was activated by estradiol followed 50 hrs later by progesterone Tests for estrus behaviour were carried out 2, 4 and 6 hrs after the progesterone injection The effects of different antidepressive substances injected 2 hrs before the administration of progesterone are presented in Fig 1

It is seen that the antidepressive compounds all inhibited the estrogen progesterone activated estrus behaviour It is also evident that this inhibitory effect decreased during the test period, being more evident during the two first tests than the last one

Desmethylimipramine seems somewhat more effective than the other substances A significant effect of trimeprimine and nortriptyline was only obtained after a comparatively high dose, 40 mg kg Furthermore, this effect was short lasting

After each test for estrus behaviour the animal tested was put on a table and the spontaneous motor activity, the gait and the exploring behaviour were observed In contrast to the inhibitory effect on the estrus behaviour of the antidepressive agents, none of the compounds tested altered the animals general behaviour There

were no signs of depression or excitation and the animals behaved completely as animals treated with hormones alone

When the higher dose levels were used mydriasis and exophthalmus were present to a variable extent

Reserpine activated estrus behaviour

A significant reduction of the estrogen reserpine activated estrus behaviour was obtained by 20 mg/kg imipramine (Table I, A) The inhibition of the heat response was 80 % at the test at 4 hrs and 60 % at the test at 6 hrs after the reserpine injection There were no or a very slight ptosis compared with animals treated with reserpine and estrogen only

The sedative effect of reserpine in this experiment was however, not or to a very slight extent counteracted by the imipramine treatment

When 20 mg/kg imipramine had been administered, 0 4 mg progesterone brought about a heat response of 35 % at 6 hrs (Table I, B) and 1 mg/kg reserpine, 33 % (Table I, A) When these progesterone and reserpine treatments were combined, 71 % of the animals went into heat (Table I, C) indicating an additive effect of the two drugs in the presence of the inhibitor The same result can be seen when the data from the 4 hr tests are compared

Tetrabenazine activated estrus behaviour

The effects of different antidepressive substances on the estrogen tetrabenazine activated estrus behaviour are presented in Figure 2

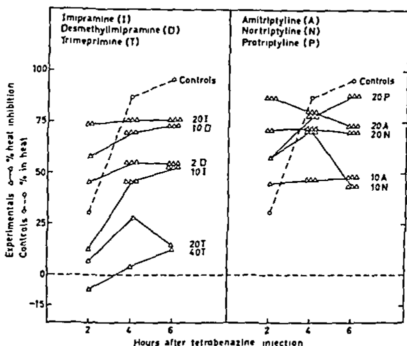
All the antidepressive substances tested except trimeprimine significantly inhibited the estrogen tetrabenazine activated heat response at the tests 4 and 6 hrs after the tetrabenazine injection (Fig 2) Trimeprimine (20—40 mg/kg) had no certain inhibitory effect in these experiments

In contrast to the progesterone experiments shown in Fig 2, the heat inhibitory effect of the antidepressive drugs was weaker as a rule at the 2 hr test than at the 4 and 6 hrs tests This latency of the inhibitory effect can be seen for example in the imipramine (10 mg/kg) experiment or the protriptyline (20mg/kg) experiment

Particular attention was paid to the signs of sedation ptosis and to the induction of compulsive exploring behaviour (see below for description) after tetrabenazine treatment alone and in combination with the antidepressive agents

The decreased motor activity, the typical sitting in a crouched position and the ptosis were fully developed 2 hrs after the administration of tetrabenazine alone The effects lasted for more than 6 hrs The results from the observations at the test 6 hrs after tetrabenazine administration are summarized in Table II Similar results were observed during the test at 4 hrs

Imipramine (20 mg/kg) injected 2 hrs before 40 mg/kg tetrabenazine almost completely prevented the development of ptosis during 6 hrs period the animals were observed after the tetrabenazine injection However no or a very slight antagonism with the sedative effect of tetrabenazine was obtained The data are in agree



Antidepressive drug mg kg		Number of animals tested	Number of runs pooled	Antidepressive drug mg/kg		Number of animals tested	Number of runs pooled
Imipramine	10	24	2	Amitriptyline	10	24	2
	20	22	2		20	23	2
Desmethyl imipramine	2	23	2	Nortriptyline	10	24	2
	10				20	24	2
Trimeprimine	20	21	2	Protriptyline	20	24	2
	40	12	1	None (controls)		24	2

The number of triangles (△—△△△) at the various points of the graph indicate the degree of statistical significance of the inhibitory effect not the number of observations

P	>0 05	0 05 0 01	0 01
Symbol	△	△△	△△△

Fig 2 The effect of different antidepressive drugs on estrus behavior in ovariectomised rats activated by tetrabenazine in combination with estradiol Estradiol benzoate 10 μg kg was injected at zero hrs followed 50 hrs later by 40 mg/kg tetrabenazine The antidepressive drugs were administered at 48 hours

TABLE II A comparison of the occurrence of ptosis sedation, compulsive exploring behaviour and estrus behaviour in ovariectomised rats after estradiol tetrabenazine treatment in combination with different antidepressive drugs

Antidepressive drug	Dose mg/kg	Ptosis	Sedation (Sitting in a crouched position decreased motor activity)	Compulsive exploring behaviour	Animals in heat at 6 hrs (%)
None	—	+++	+++	0	96
Imipramine	10	+++	++	0	41
	20	+	++ or ++	0	21
Desmethylimipramine	2	0	+ or ++	+	44
	10	0	0	+++	26
Trimeprimine	20	+++	+++	0	81
	40	+++	++ or	0	83
Amitriptyline	10	++	+++	0	50
	20	++	+++	0	26
Nortriptyline	10	0	0	+	54
	20	0	0	+++	19
Protriptyline	20	0	0	+	21

Estradiol benzoate 10 μg/kg was injected at zero hours Tetrabenazine 40 mg kg and the antidepressive drug were administered at 48 and 50 hours respectively
The observations were made six hours after the tetrabenazine injection The number of runs and of animals tested can be seen from the text to fig 2 and 3
Response graded from none (0) trough slight (one symbol) to obvious (three symbols

ment with those of Sulser *et al* (1962), who found that 20 mg kg imipramine i p reversed the ptosis activated by a benzoquinolizine amine depletor (Ro 4—1284 related to tetrabenazine, but did not prevent the sedative effect of this amine depletor Thus, in spite of the fact that 10—20 mg kg imipramine did not remove the sedative effect of tetrabenazine, the frequency of animals displaying mating behaviour after estrogen in combination with tetrabenazine was clearly reduced by this drug

A significant reduction of the tetrabenazine induced heat response was obtained at the 4 and 6 hrs tests by 2 mg/kg desmethylimipramine Fig 2) The sedation in these experiments varied Some animals were slightly sedated while other animals were sedated to almost the same extent as when only tetrabenazine had been given No relationship ($p > 0.1$) was seen between sedation and estrus behaviour i e the animals in heat were neither more nor less sedated than the animals not in heat No animals had ptosis (Table II

When the dose of desmethylimipramine 2 hrs before 40 mg kg tetrabenazine, was increased to 10 mg/kg in an analogous experiment the peculiar behaviour described by Sulser *et al* (1962) was observed When put on a table the animals displayed hyperactivity characterized by circling along the table edge, and i

Table III The effect of combined treatment with imipramine and monoamine oxidase inhibitors on estrus behaviour in ovariectomised rats activated by estradiol in combination with progesterone

Estradiol benzoate, 10 µg/kg was injected at zero hours followed 50 hours later by 0.4 mg/animal progesterone

Treatment	Results						
		Animals in heat				Number of	
			Experimentals				
At 48 hrs	Hours after progesterone injection	Controls %	%	% inhibition	P for difference between expts and controls	animals tested	runs pooled
A Imipramine, 10 mg/kg (controls had hormones alone)	2	34	17	52	<0.05		
	4	69	63	9	>0.05	32–35	3
	6	78	83	–6			
B Imipramine 10 mg/kg and nialamide 100 mg/kg (controls had nialamide alone)	2	35	0	100	<0.01		
	4	83	38	54	<0.01	23–24	2
	6	83	54	35	>0.05		
C Imipramine 10 mg kg and nialamide 200 mg/kg (controls had nialamide alone)	2	17	0	100	>0.05		
	4	50	9	82	<0.05	12	1
	6	50	9	82			
D Imipramine 10 mg kg and pargyline 25 mg kg (controls had pargyline alone)	2	29	9	69	0.05		
	4	43	13	70	0.03	21 23	2
	6	57	17	70	0.02		

Treatment A values taken from fig 2

exploring behaviour was directed towards and over the table-edge, so that they fell to the floor This behaviour was repeated over and over again In the following this hyperactivity will be described according to Bickel *et al* (1963) as a "compulsive" exploring behaviour In spite of this behaviour, it was possible to perform the tests for estrus behaviour when the animals were transferred to the male cages A slight compulsive exploring behaviour was also seen in the 2 mg kg expt (Table II) Left alone in their cages most animals displayed the usual reserpine like sedation sitting in a crouched up position In a few cases, however when handled these animals started the non-stop circling around

The compulsive exploring behaviour was not seen in the imipramine, trimeprimine and amitriptyline experiments but was displayed only by animals treated with the monomethyl derivatives in combination with tetrabenazine The monomethyl deriv-

atives i e desmethylimipramine nortriptyline and protriptyline, also prevented the development of ptosis and sedation more actively than the dimethyl derivatives However such an obvious difference between the two classes of substances concerning the effect on the estrogen tetrabenazine activated estrus behaviour was not obtained

The effect of imipramine in combination with monoamine oxidase inhibitors

In order to investigate if inhibition of the monoamine oxidase activity with subsequent increase of the monoamine levels would give a synergistic effect to that of imipramine the monoamine oxidase inhibitors nialamide or pargyline were administered together with imipramine (Table III) Estrus behaviour was activated by estrogen in combination with progesterone

Imipramine (10 mg/kg) administered 2 hrs before the progesterone treatment did not reduce the heat response at 4 and 6 hrs after the progesterone administration (Table III, A) An obvious reduction of the heat response was obtained however, after combined treatment with this dose of imipramine and the monoamine oxidase inhibitors (Table III, B, C, D) indicating a synergistic effect

Atropine and estrogen progesterone activated estrus behaviour

Since imipramine has atropine like effects (Sigg 1959 b Halliwell Quinton Williams 1964) it was of interest to study the effect of atropine on estrus behaviour

Atropine 1 mg/kg was injected similarly to the antidepressive substances 2 hrs before the progesterone treatment Tests for estrus behaviour were carried out at 2 4 and 6 hrs after the progesterone injection A pronounced mydriasis but no inhibitory effect on the estrogen progesterone activated estrus behaviour was seen

Discussion

Imipramine clearly inhibited the estrus behaviour activated by reserpine or tetrabenazine in combination with estrogen Thus the well known ability of imipramine to counteract the central symptoms of reserpine and reserpine like drugs is true also for the estrus behaviour activating effect of reserpine and tetrabenazine

It has been shown by Axelrod *et al* 1962 that imipramine interferes with the uptake of catecholamines in tissues From the investigations of Carlsson and Waldeck (1965 a b) and Malmfors (1964) there is biochemical and histochemical evidence that imipramine and related thymoleptic drugs prevent the amine uptake in adrenergic nerves by inhibition of the cell membrane transport mechanism By means of a combination of biochemical and histochemical techniques it has been possible to show that this is probably also valid for central nervous catecholaminergic neurons (Carlsson *et al* 1965) The blocked amine uptake would result in increased availability of catecholamines at the receptors with subsequent increased central adrenergic tone

Direct functional data that imipramine potentiates central nervous adrenergic mechanisms according to the original suggestion of Sigg 1959) are rather Everett (1965) has reported that imipramine amitriptyline and its demet

derivatives potentiate the stimulating effect of DOPA in reserpine treated animals and similar results have been described by Sulser *et al* (1964)

The fact that hormone activated estrus behaviour was inhibited by increased monoamine levels (Meyerson 1964 a b) makes it reasonable to assume that the inhibitory effect of imipramine on estrus behaviour is due to increased activity at monoaminergic central synapses This is also indicated by the inhibitory effect achieved by the combination of imipramine and the monoamine oxidase inhibitors pargyline and nialamide Imipramine lacks monoamine oxidase inhibitory properties (Pulver Exer, Herrmann 1960) The effect of the combination is conceivably due to increased intraneuronal monoamine levels with subsequent leakage to the receptors and decreased pumpback mechanism to the nerve terminals

There is however, good evidence from previous investigations that the heat inhibitory monoaminergic pathways are mainly serotoninergic The inhibitory effect of monoamine oxidase inhibitors was obviously potentiated by subsequent administration of the serotonin precursor 5 hydroxytryptophan Estimation of serotonin and catecholamines in brain tissue of rats treated with hormones and pargyline and tested for estrus behaviour showed that the heat inhibitory effect of increased monoamine levels was more closely correlated to increased serotonin levels than to the increase of catecholamines (Meyerson 1964 b) It was also demonstrated that the inhibitory effect on estrus behaviour of pargyline was prevented by the substance α propyl-dopacetamide (H 22 54) which inhibits serotonin synthesis It is therefore likely that imipramine inhibits estrus behaviour mainly by potentiating central serotoninergic functions

Little is known about the effect of imipramine and related compounds on central nervous serotoninergic functions A potentiation of serotoninergic functions on peripheral receptors is indicated by the increased effect of serotonin on the cat nictitating membranes after imipramine treatment Gyermek and Possemato 1960 This action was further confirmed and similar effects of other antidepressive drugs demonstrated by Sigg Soffer and Gyermek 1963

The view that serotonin rather than catecholamines is involved in the inhibitory effect on estrus behaviour of the antidepressive drugs is supported by the failing correlation between the ability of the different compounds to inhibit estrogen tetrabenazine activated estrus behaviour and the ability to prevent tetrabenazine induced sedation and to induce compulsive exploratory behaviour in tetrabenazine treated rats

Accumulating evidence indicates that the sedative state induced by reserpine or tetrabenazine is due to impaired catecholaminergic transmission (Carlsson Lindqvist Magnusson 1957 Everett and Wiegand 1962 Carlsson 1964) Sulser *et al* (1964) have brought forward evidence that desmethylimipramine brings about the characteristic compulsive exploring behaviour by mediating an increased catecholaminergic tone

Imipramine and amitriptyline inhibit the estrus behaviour in doses which do not prevent tetrabenazine induced sedation or induce compulsive exploratory behaviour

These antidepressive drugs thus might possibly increase serotoninergic tone more actively than the catecholaminergic one Desmethylimipramine would seem to act on catecholaminergic and serotoninergic mechanisms to roughly the same extent as a fairly good relationship is here seen between the effect on estrus behaviour and the one on sedation and compulsive exploratory behaviour

Not only the estrus behaviour activated by amine depletors but also that activated by progesterone was clearly inhibited by the antidepressive compounds

A comparison of the progesterone and tetrabenazine experiments reveals that the inhibitory effect of the antidepressive substances in the progesterone experiments was more evident in the earlier tests (at 2 and 4 hrs after progesterone treatment) while the opposite was the case in the tetrabenazine experiments where a certain latency was present In this respect reserpine seems more alike progesterone (Table I)

The present data also show that the heat inhibitory action of imipramine was overcome when progesterone and reserpine treatment were combined, which indicates a synergistic action on estrus behaviour of these two drugs

Imipramine has anticholinergic properties It is not likely, however, that its action on estrogen progesterone activated estrus behaviour is an atropine like anticholinergic effect No inhibition was seen after 1 mg/kg atropine This dose has central effects The duration of tremor induced by tremorine, a substance with cen -al cholinergic effects (Everett, Blockus and Sheppard 1956), was shortened by 0 5 ig kg atropine in rats (Sjoqvist and Gilette 1965) Furthermore, desmethylimipra iine excerted a greater inhibitory effect than imipramine but has weaker anti holinergic properties (Halliwell *et al* 1964)

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Acta physiol. scand. 1966. 67. 423—429
From the Department of Pharmacology, University of Goteborg Sweden

Uptake of Metaraminol by the Adrenal Medullary Granules

By

PER LUNDBORG

Abstract

LUNDBORG, P *Uptake of metaraminol by the adrenal medullary granules* Acta physiol scand 67 423—429

Metaraminol is taken up by the adrenal medullary granules *in vitro* by displacing the endogenous catecholamines from their storage sites This uptake is not blocked by reserpine Metaraminol inhibits the ATP Mg dependent uptake of adrenaline but does not utilize this mechanism for uptake There thus seem to exist two uptake mechanisms in the amine granules *i e* one ATP Mg -dependent mechanism sensitive to reserpine and the other acting by displacement of endogenous amines Dopamine is capable of utilizing both and metaraminol only the latter mechanism

Independent work by Carlsson, Hillarp and Waldeck (1962) and Kirshner (1962 a, b) has shown that the amine granules of the adrenal medulla are able to take up and concentrate monoamines *in vitro* by a Mg^{++}-ATP-dependent storage mechanism The mechanism is blocked by reserpine in low concentrations Euler and Lishajko (1963) have discovered a Mg^{++}-ATP dependent uptake of noradrenaline also by the amine granules of the adrenergic nerves Also this uptake was blocked by low concentrations of reserpine

In the present paper the interaction of metaraminol with the Mg^{+} -ATP-dependent uptake mechanism in the adrenal medullary granules is examined

Material and methods

Amine granules from cow adrenal medullas were prepared essentially as described by Hillarp (1958). The medulla was homogenized with a loose fitting plastic pestle in 0 3 M sucrose Unbroken cells and nuclei were removed by centrifugation at 800 g for 5 min The supernatant was decanted and centrifuged at 26 000 × g for 20 min The supernatant was decanted and the looser layer above the more tightly packed bottom sediment was removed by swirling with sucrose Finally the granules were suspended in 0 3 M sucrose and stored at 0 They were used the same and the next two days

Standard conditions for the experiments An aliquot (50 μl) of the granule suspension in most experiments correspond

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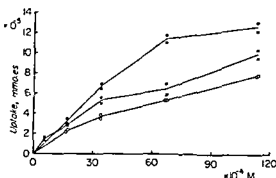


Fig 1 Effect of various concentrations of (—)-metaraminol on the uptake of (±) H^3-metaraminol Three experiments with three different samples of granules The specific activity of metaraminol was constant at the different concentrations

rinsing of the tubes with 0 5 M sucrose the granule sediment was extracted with 5 0 ml of 0 01 N HCl in 98 % ethyl alcohol In some experiments the catecholamine content of the extracts was determined spectrophotofluorimetrically (Bertler, Carlsson and Rosengren 1958) The C^{14} or H^3 content of the extracts was determined directly in a liquid scintillation counter Using the present experimental conditions Carlsson, Hillarp and Waldeck (1963) examined, after incubation with C^{14} dopamine or C^{14}-adrenaline, the C^{14}-labelled compounds of the extracts by paper chromatography followed by scanning the chromatogram for radioactivity Using labelled adrenaline C^{14} was present only in the adrenaline spot When C^{14} dopamine was used C^{14} was present mainly in the dopamine spot but also in the noradrenaline spot (10 %)

Substances used (—)-metaraminol bitartrate was generously supplied by Merck, Sharp and Dohme, Dr K C Mezey (±)-H^3-metaraminol was prepared by the research laboratory of Hässle Ltd in cooperation with this laboratory (see Carlsson and Waldeck 1965) (+) metaraminol bitartrate was generously supplied by Hässle Ltd, Dr H Corrodi Commercially available C^{14} tyramine, C^{14}-dopamine and (±) C^{14} adrenaline were used Reserpine was generously supplied by Swedish Ciba Ltd Stockholm Sweden

Results

Uptake of metaraminol as function of external amine concentration

With increasing concentration of metaraminol in the medium the absolute uptake increased almost linearly up to a concentration of about 1,000 μg metaraminol bitartrate/ml (about 3×10^{-3} M), above this uptake it increased more slowly (Fig 1) At calculation of the absolute uptake the values were multiplied by the ratio of unlabelled (—)-metaraminol to H^3-metaraminol in the medium This may give somewhat too low values on account of a preferential uptake of the (—)-form However, the uptake mechanism does not seem to be saturated until above 2×10^{-2} M When Carlsson *et al* (1963) studied the Mg -ATP dependent uptake of catecholamines, they noticed that this uptake mechanism seemed to be saturated already at 4—5 $\times 10^{-4}$ M This might indicate that metaraminol has a much lower affinity to the Mg -ATP-dependent uptake, or that we are dealing with another mechanism

Inhibition of the ATP-Mg -dependent uptake of adrenaline by metaraminol

When adrenaline in an external concentration of about 30 μg ml (25 μg unlabelled (—)-adrenaline, 5 37 μg C^{14}-adrenaline), (30 μg ml $\sim 1\,6 \times 10^{-4}$ M) is incubated together with metaraminol in increasing concentration, a gradually increasing inhibition of the adrenaline uptake is observed (Fig 2) To study the nature of this

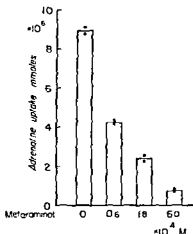


Fig 2 Inhibition of adrenaline uptake by metaraminol ( ) C14 adrenaline 5·01 µg unlabelled (—) adrenaline ?? µg To the incubation vessels was added (—) metaraminol in varying concentrations

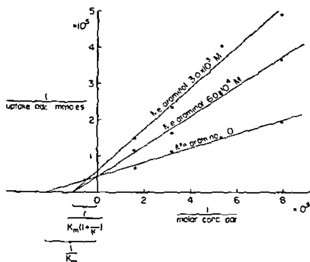


Fig 3 Effect of various concentrations of adrenaline on the uptake of C14 adrenaline in the presence of (—)-metaraminol The specific activity of adrenaline was kept constant

inhibition various amounts of adrenaline were incubated together with various concentrations of metaraminol The values obtained were plotted according to the method of Lineweaver and Burk (1934) as described by Jonasson Rosengren and Waldeck (1963) It is assumed, as said by Jonasson *et al*, that there is a reaction of the following type



$$A_1 + S \rightleftarrows A_1S \rightarrow A_2 + S$$

Where A_1 = free amine, A_2 = bound or conjugated amine and S — the hypothetical "transfer site" of the granules necessary for amine incorporation From Fig 3 it can be calculated that K_m for the adrenaline uptake is $4 \cdot 3 \times 10^{-4}$ This value is somewhat lower than the K_m value found by Jonasson *et al* (8×10^{-4} but in other experiments we have
may vary with different gr

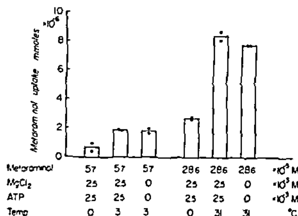


Fig 4 Influence of ATP Mg and temperature on H^3 metaraminol uptake Different granule samples were used for the two different concentrations of metaraminol

aminol in a concentration of 200 μg/ml ($\simeq 6 \times 10^{-4}$ M) acts mainly as a competitive inhibitor The K_i value for metaraminol can be calculated

$$\frac{1}{K_m\left(1 + \frac{1}{K_i}\right)} = 1\,08 \times 10^3$$

$$K_i = 5\,2 \times 10^{-4}$$

This indicates that there is no appreciable difference between metaraminol and adrenaline as regards the affinity to the ATP Mg dependent uptake mechanism in the granules At a higher concentration of metaraminol in the incubation medium (Fig 3 upper curve) a curve indicating mixed inhibition was obtained This might be explained by the fact that even if metaraminol is a competitive inhibitor of the Mg ATP dependent uptake of adrenaline it is probably taken up itself by another mechanism see Discussion) thereby presumably displacing some of the C^{14} adrenaline already incorporated

Influence of Mg and ATP temperature and reserpine on the uptake of metaraminol

In some experiments metaraminol at low concentrations (about 19 or 96 μg/ml), ($\simeq 0\,57 \times 10^{-4}$ M and $2\,9 \times 10^{-4}$ M resp) was incubated according to Fig 4 Every column represents the mean of two values From the figure it appears that there exists a temperature dependent uptake of metaraminol It also appears that there is no or at most a slight decrease in the uptake of metaraminol if Mg and ATP are excluded

In some other experiments metaraminol was incubated together with reserpine in varying concentrations As shown in Fig 5 reserpine even in rather high concentration, had no influence on the accumulation of metaraminol in the granules

Thus the incorporation of metaraminol into the granules differs in many respects from that of adrenaline and other catecholamines It is not influenced by the addition of ATP and Mg It is not blocked by reserpine Furthermore the uptake

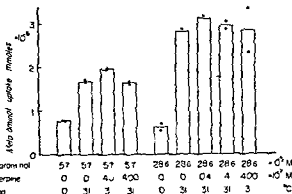


Fig 5 Failure of reserpine to influence the uptake of metaraminol

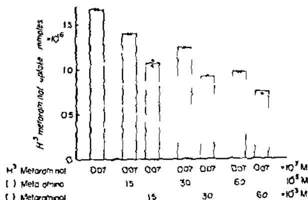


Fig 6 Influence of varying concentrations of (—) and (+) metaram nol on H³ metaram nol uptake

mechanism is not saturated until there is a concentration of metaraminol above 2×10^{-3} M

Uptake of (—)- and (+) metaraminol

H³ metaraminol was available only in the (±) form Therefore an indirect approach was used to study the stereospecificity of the uptake mechanism Granules were incubated in a medium containing a constant concentration of H³ metaraminol To this medium were added varying concentrations of unlabelled (—)- or (+) metaraminol Fig 6 shows the results of a typical experiment The results are expressed as means of two values, which show very good agreement As seen in the figure the dilution with the (—) form reduced the uptake of H³ metaraminol more than dilution with the (+) form The most probable explanation for this is that the uptake is not stereospecific but has preference for the (—) form

The concomitant uptake of metaraminol and release of adrenaline and noradrenaline

Carlsson and Hillarp (1961) have suggested the existence of an uptake mechanism in the adrenal granules, where the incorporation mainly represents an exchange between the stored adrenaline + noradrenaline and the added amines Schumann (1961) and Schumann et Philippu (1961, 1962) have given evidence for the view

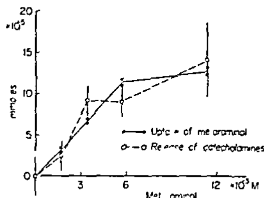


Fig 7 The concomitant uptake of metaraminol and release of catecholamines at varying external metaraminol concentrations Release of catecholamines was determined as the difference between samples and controls The *bars indicate s e of the difference (n = 2)* Metaraminol data same as in *Fig* 1 upper curve

TABLE I Uptake of metaraminol tyramine and dopamine The concentration of the amines were in all cases 5 5 × 10 $^{-4}$ M

Amine	Uptake of amines mmoles × 10 $^{-8}$
Metaraminol	15 4
	16 8
Tyramine	25 0
	25 8
Dopamine	18 3
	17 9

that tyramine *in vitro* gives a release of the catecholamines in the storage granules and that tyramine causes this release by displacing the amines from their storage sites To settle if metaraminol is incorporated in the same way, metaraminol was added to the incubation medium in increasing concentration As shown in Fig 7, there is a rather good correlation when expressed in mmoles between the uptake of metaraminol and the concomitant release of adrenaline + noradrenaline from which might be suggested that also metaraminol is taken up by displacing the amines from their storage sites In Table I is shown that the uptake of metaraminol, dopamine and tyramine is of about the same order of magnitude, which supports the *suggestion that these amines when added in high concentrations are utilizing* the same mechanism for accumulation in the granules

Discussion

Bringing all these data together it can be assumed that metaraminol is incorporated into the amine granules by a mechanism which is not or at most slightly utilizing Mg^{++} and ATP The mechanism is temperature dependent and is not influenced by reserpine It is not saturated until at high concentrations — above 2 × 10 $^{-4}$ M Metaraminol in lower concentrations is a competitive inhibitor of the Mg^{++}-ATP dependent uptake of adrenaline in the granules Its K_i value is almost the same as the K_m value for adrenaline indicating that metaraminol and adrenaline have almost the same affinity to the Mg^{++} ATP-dependent uptake mechanism, though

metaraminol does not utilize this mechanism for accumulation in the grar Furthermore, the experiments indicate that metaraminol is displacing the end nous catecholamines from their storage sites in the chromaffin granules

It may be concluded that there exist at least two different mechanisms for accun u lation of amines in the granules, 1) the ATP Mg dependent uptake, which i blocked by reserpine and 2) the displacing mechanism Metaraminol and tyramine are able only to utilize the displacing one, while, for example, dopamine is able to utilize both mechanisms In lower concentrations the Mg ATP dependent uptake is the most efficient, dominating the dopamine uptake but in concentrations above 5 $\times 10^{-4}$ M, when this mechanism is saturated, dopamine is taken up only by displacement

The ability of the rat heart to retain metaraminol *in vivo* is considerably blocked by reserpine (Shore, Busfield and Alpers 1964, Carlsson and Waldeck 1965) Further investigations are required to elucidate this apparent discrepancy between *in vitro* and *in vivo* data

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Acta physiol. scand. 1966. 67. 430—440

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Release and Uptake of Noradrenaline in Adrenergic Nerve Granules

By

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Abstract

Euler, U. S. v. *Release and uptake of noradrenaline in adrenergic nerve granules.* Acta physiol. scand. 1966. 67. 430—440.

A brief review is given of some recent findings concerning noradrenaline (NA)[1] release and uptake in isolated nerve granules and in connection with neurotransmission *in vivo*. The main results are summarized in the following points. Rabbit heart granules have similar NA release rate and other properties as bovine nerve granules. Granules from the bovine vesicular gland show about 3 times lower NA release rate and differ in other respects from splenic nerve granules. Tyramine [illegible] and [illegible] overcome by "neuronal rest" and injection of NA or dopamine. Although homogenization experiments indicate that most of the NA in axones and nerve endings occur in granule bound form it is assumed that part of the NA is present as "transport NA" in loosely bound form available for immediate release.

Since the demonstration of specific adrenergic transmitter granules in nerves and organs (Euler and Hillarp 1956) a large amount of work has been directed towards the study of factors which govern the release and uptake of noradrenaline (NA) in such granules. From these studies it has emerged that bovine splenic nerve granules, on which most of the experiments have been made, have a characteristic release with a half-time of about 10 min at 37° and pH 7.5 which clearly distinguishes them from adrenal medullary granules. The release rate is greatly influenced by temperature, pH in the medium, the presence of NA in the incubation medium and by addition of ATP or ADP (Euler and Lishajko 1963 a, b). A large number of drugs also influence the release rate in a characteristic way, either increasing or decreasing the release rate (cf. Euler and Lishajko 1965 a). After partial NA depletion isolated granules take up NA and other catecholamines. This uptake is greatly enhanced by addition of ATP-Mg to the incubation medium (Euler and Lishajko 1963 b).

[1] Abbreviations used: A (a[illegible]), NA (noradrenaline), PBA (phenoxybenzamine), DCI (dichloroisoproterenol), ATP ([illegible]phosphate).

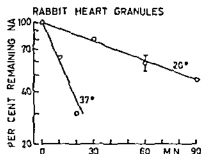


Fig 1 Release of NA from isolated rabbit heart granules in phosphate buffer pH 7 5 at 20° and 37°

TABLE I Rabbit heart granules Remaining NA in per cent of original with addition of NA and ATP to the incubation medium Incubation time 60 min 20

Control not incubated	100
Control incubated	59
+NA 10 μg/ml	71
+ATP 3 mM	65
+NA+ATP	81

Properties of granules of different origin

Rabbit heart

The NA release curve for resuspended rabbit heart granules obtained by homogenization of the organ and high speed centrifugation after removal of coarse material follows an exponential course with an average half time value of 13 min at 37° (Fig 1)

At 37° the release rate is about 6 times higher than that at 20°, showing a similar high temperature dependence as for bovine splenic nerve granules Changing the medium from phosphate to sucrose does not appreciably alter the release rate as noted also for nerve granules

In a concentration of 10 μg ml NA exerts a "protective effect depending on a compensatory NA uptake (Euler Stjarne and Lishajko 1963) though less marked than for nerve granules

The small effect on the NA release rate on addition of ATP in a 3 mM concentration is in contrast to the results on splenic nerve granules where ATP Mg exerts a marked "protective effect even in resuspended granules without addition of NA It is uncertain however whether this difference is real since the amount of tissue material in the heart granular fraction is considerably larger per μg NA than in nerve splenic granules Adsorption or breakdown of ATP might therefore occur to a greater extent In addition the NA concentration in the medium may be subthreshold or suboptimal

If both NA and ATP are added to rabbit heart granules the protective effect is more marked than with NA alone Moreover the effect is dependent on the concentration of NA and is smaller at 2 μg/ml NA than at 10 μg ml Table I shows

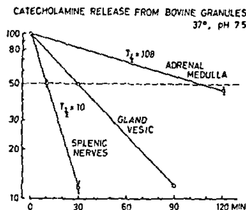


Fig 2 Release of NA from isolated granules from bovine adrenal medulla vesicular gland and splenic nerves in phosphate buffer pH 7 5 at 37°

the effect of addition of NA alone, ATP alone and NA + ATP on the amount of NA remaining in the granules after 60 min incubation at 20° An actual uptake of NA as well as A also occurs after partial depletion of the NA in the heart granules in the presence of ATP

The characteristic lowering of the release rate on addition of reserpine in concentrations of 10 ⁴—10 ⁴ M to the incubation medium does not appear with isolated rabbit heart granules However, in granules prepared from the hearts of rabbit, which had received a dose of 0 25 mg/kg reserpine i v 10 min previously, a slower lease rate is noted

The results recorded with rabbit heart granules, based on direct amine analysis are in good agreement with many of those obtained by Potter and Axelrod (1963 on rat heart granules with radioactive NA

Vesicular gland and vas deferens of the bull

It has been shown earlier that the vesicular gland and the vas deferens of the bull contain relatively large amounts of an adrenaline-like substance later shown to be noradrenaline (Sjostrand 1965 which occurs in amounts up to about 10 μg g tissue, and thus is higher than in any other organ except the adrenal medulla and paraganglia From the studies of Sjostrand 1965 it has emerged that section and degeneration of the hypogastric nerves causes only a moderate reduction of the noradrenaline content in the vas deferens of the guinea pig and in male accessory glands of several other animal species By using the fluorescence technique of Falck and Hillarp it has been established that the NA in these organs occurs in short adrenergic neurons (Owman and Sjostrand 1965

A study of the NA granules in the vesicular gland prepared in the same way as for the rabbit heart, has shown that they have properties which in several respects differ from those of the splenic nerves Thus the release rate for NA is considerably lower in the vesicular gland granules which show a half-time of about 30 min as compared to about 10 min for splenic nerve granules at 37 Euler and Lishajko 1966 a) The exponential course of the NA release also shows that the release rate is

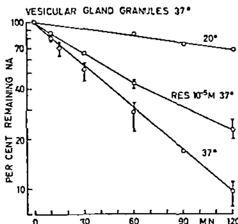


Fig 3 Release of NA from isolated bovine vesicular gland granules in phosphate buffer pH 7 5 at 20° and 37° Effect of reserpine 10 ^{5} M in incubation medium at 37°

approximately constant, indicating that the observed function is not a resultant of the release from nerve granules with a rapid release and others with a low release rate such as e g chromaffin cell granules (Fig 2)

The deviating NA release rate of the granules from the vesicular gland suggests that these may differ from other nerve granules also in other respects On addition of ATP (3 mM) to the incubation medium a certain "protective" effect on the NA release can be seen as in splenic nerve granules, but only to a small extent, the half-time increasing from 34 min to 43 min The "protective" effect of NA alone (6 × × 10 ^{5} M) is likewise quite small and in no way comparable to that on splenic nerve granules

The NA uptake from the medium containing NA (6 × 10 ^{5} M) in partially depleted granules from the vesicular gland is almost negligible in 30 min at 20 even in the presence of ATP (3 mM) which in splenic nerve granules causes an uptake almost to the original amount of NA The uptake of A is equally unconspicuous as that of NA

Tyramine and octopamine in a concentration of 6 × 10 ^{5} M both slightly increase the release rate but less than for splenic nerve granules Of other drugs reserpine lowers the release rate in a concentration of 10 ^{5} M Fig 3

PBA inhibits the NA release from vesicular gland granules much less than for splenic nerve granules and the same difference is noted also for prenylamine (Segontin[8]) 3 × 10 ^{5} M When the same drugs are added to sedimented and resuspended vesicular gland granules, no inhibitory action is observed Propranolol, which in the concentration 3 × 10 ^{4} M strongly inhibits the NA release from splenic nerve granules (Euler and Lishajko 1966 b) even causes a moderate increase of the NA release rate from vesicular gland granules

Granules prepared from the vas deferens of the bull also show a considerably slower release rate than splenic nerve granules, the half time at 37 and pH 7 5 in the phosphate buffer medium being about 25 min

The results with granules from the vesicular gland of the bull thus clearly indicate that these granules have some properties which differ considerably from those of other bovine nerve granules, such as from the splenic nerves The differences can only to a minor part be ascribed to the preparatory technique since granules obtained by squeezing the nerves or by homogenization with a Turrax apparatus behave in a similar way as regards the release rate, in spite of the fact that the granule suspension in the former case contains much less of broken particles and tissue material than the homogenized nerve

Since hardly anything is known about the mechanism for the action of various drugs on the release of NA from the granules it does not appear fruitful at present to discuss the difference in action of drugs on granules of different origin It is so far wholly unexplained why for instance PBA inhibits the NA release from splenic nerve granules and in the same concentration range, 1—4 × 10^{-4} M, strongly enhances the catecholamine release from adrenal medullary granules from the same species and in the same medium while hardly any actions is observed on vesicular gland granules Equally little is known about the true reason for the different spontaneous release rates between the three kinds of granules In this context it is perhaps pertinent to draw attention to the relative release rates of amines and ATP, which differ very markedly between splenic nerve granules and adrenal medullary granules While the amine ATP ratio remains largely unchanged in adrenal medullary granules on incubation Hillarp 1958) this ratio falls conspicuously in splenic nerve granules (Euler Lishajko and Stjarne 1963)

Drug action on isolated NA granules

It is hardly surprising that drugs with marked actions on membranes or interfering with basic metabolic actions such as enzyme poisons should influence the release or uptake of NA in granules Actions of this kind may give some clues as to the kind of chemical or physico-chemical processes underlying the release and uptake processes No precise informations have been obtained so far however since it is difficult to ascertain whether a NA releasing action for instance by dinitrophenol on nerve granules, is a specific action or not cf Euler and Lishajko 1965 a

Tyramine

In some instances the effect of drugs on nerve granules depends on uptake on a competitive basis which primarily involves certain mono and diphenolic amines Effects of this kind in many cases represent the underlying mechanism for the "false transmitter" phenomenon cf Carlsson and Lindqvist 1962)

As originally shown by Schumann 1960 tyramine causes a release of catechol amines from isolated granules Recently Muscholl 1965 and others have produced evidence to show that tyramine inhibits the reuptake of NA *in vivo* If tyramine causes the same kind of effect on isolated granules by competing with NA the apparent release of NA would also be increased Fig 4 shows an experiment with splenic nerve granules in which tyramine added in approximately the same concentration

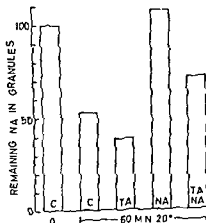


Fig 4 Effect of tyramine (TA) and NA 6×10^{-4} M, on release of NA from bovine splenic nerve granules in phosphate buffer at pH 7 5 incubated 60 min at 20°

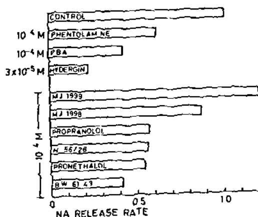


Fig 5 Effect of various α and β blockers on release rate (control = 1) of NA from bovine splenic nerve granules incubated in phosphate buffer pH 7 5 60 min at 20°

as NA (6×10^{-6} M) to the medium causes a loss of NA, presumably by inhibiting the compensatory uptake of NA which normally occurs when this amine is present in the medium The "protective" effect of added ATP Mg on the NA release is likewise counteracted by tyramine Tyramine also inhibits the uptake of NA in partially depleted granules in the presence of ATP Mg The degree of inhibition is dependent on the concentration ratio tyramine NA

Adrenergic receptor blockers

An inhibitory action of PBA on the NA release from bovine splenic nerve granules has been demonstrated earlier Euler and Lishajko 1965 a) Subsequent studies have shown that other α-blockers such as phentolamine and Hydergin also inhibit the NA release

A number of β blockers were even more effective in this respect Thus DCI, pronethalol, propranolol and H 56 28 in the concentration range 3×10^{-5}—$3 \times$ $\times 10^{-4}$ M decreased the release rate to about one half of that in the control (Fig 5) (Euler and Lishajko 1966 b)

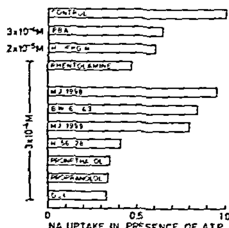


Fig 6 Effect of various α and β blockers on NA uptake in the presence of 3 mM ATP Mg in partially depleted bovine splenic nerve granules incubated in phosphate buffer pH 7 5 30 min at 20°

In this connection it may be of interest to mention that an uptake of isoprenaline occurs in partially depleted nerve granules to about the same extent as NA or A (Euler and Lishajko, unpubl expts)

The inhibitory action on the NA release prompted a study of the action of the drugs on the capability of the isolated granules to take up NA after partial depletion The effect of the blockers on the NA uptake was tested in the presence of ATP Mg which normally greatly enhances the uptake process in splenic nerve granules As a ... lt of these experiments it was found that all the adrenergic blockers tested inhib ... the NA uptake Fig 6 Of the drugs used DCI, pronethalol propranolol and H 56 28 are particularly active, while MJ 1998 and 1999 and N isopropyl methoxamine have only a weak action in this respect

It has been suggested that the relatively weaker effects of MJ 1998 and 1999 in vitro as compared with those in vivo depend on the low degree of lipid solubility (Kvam, Riggilo and Lish 1965 With regard to N isopropyl methoxamine its effect appears to be low in vivo in comparison with DCI Hardman Barboriak and Meester 1963

These results show that a variety of adrenergic blocking substances have properties in common on isolated nerve granules viz 1 to inhibit the amine release from nerve granules and 2 to counteract the NA uptake in the presence of ATP Even if no conclusions are warranted so far as to the mode of action of the adrenergic blockers tested or whether the two effects are related to each other or not the results make it appear possible that the blocking activity of the drugs on receptors is in some way related to an amine uptake mechanism with characteristics which are common to the target cells and the granules The nature of this blockade is unknown although the possible participation of an ATP-dependent mechanism should be considered In this context it should be recalled that Belleau 1960 has considered ATP as an integral part of the adrenergic receptor The results reported also seem to make it probable that part of the "sympatholytic" effect observed in vivo with adrenergic blockers may be due to the inhibitory effect on transmitter granule functions Thus

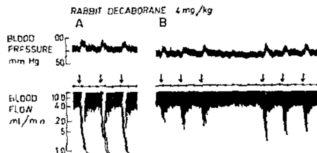


Fig 7 Rabbit nembutal treated with decaborane 4 mg/kg i p 24 hrs previously Effect of lumbar sympathetic stimulation 30 sec 20 imp sec at 1 min intervals A Initial effects of stimuli B 90 min later effect of prolonged stimulus interval

the interaction between blocker and NA or A uptake on isolated nerve granules may have its counterpart in competitive blocking *in vivo*, since an increase in the amine concentration reduces the inhibitory effect of the drug on the granules in vitro (Euler and Lishajko 1966 b)

Transmission failure following depletion of NA stores in vivo

In recent years a number of substances have been found to cause depletion of the NA stores in vivo Several of these appear to replace the normal transmitter, while others like reserpine, seem to interfere with more than one process of importance for maintaining the normal amount of transmitter in the granules (cf Stjarne 1966)

Among the substances capable of depleting the NA stores, the boron hydride decaborane (B_{10} H_{14}) appears to occupy a special position Depletion of the NA stores in the brain of rats after decaborane was described by Merritt, Schultz and Wykes (1964) and it was later shown that it caused a profound lowering of the NA content in a variety of organs even after administration of doses as small as 4 mg/kg intraperitoneally The effect was slow in onset and the fall in NA was most marked 24—48 hrs after the injection It was also shown (Euler and Lishajko 1965 b) that injections of NA promptly refilled the NA stores of the heart in the rabbit and that the NA taken up gradually left the stores over a period of several hours During this time the NA was normally distributed between the particulate and the soluble fraction separated by high speed centrifugation after homogenization of the organ Decaborane 10^{-6} M and stronger also increased the release rate of nerve granules in vitro The results appear to indicate that the amine uptake mechanism is relatively efficient after decaborane The gradual disappearance of the transmitter from the endogenous stores or of the NA taken up after injection might indicate either a slight increase in the release rate or a normal release not compensated for by normal reuptake or resynthesis

A deficiency in sympathetic nerve transmission presumably owing to lack of transmitter has been demonstrated after reserpine and other depleting drugs As shown by Burn and Rand (1958) and by Rosell and Sedvall (1961) administration of NA or A by i v injection or infusion can temporarily restore the nerve transmittor function

In a study on decaborane-depleted rabbits Bygdeman and Euler (1966) shown that intermittent trains of stimulation to the lumbar sympathetic ch

30 to 60 sec duration at a frequency of 20/sec and 1—2 min interval causes a *reduction* of the vasoconstrictor response in the perfused hind leg The deficiency usually became evident after some 6—12,000 stimuli delivered in 10—20 series of stimuli No transmission deficiency over several hours was noted in control animals, *indicating that* the decreasing response (Fig 7) was not due to conduction failure caused by prolonged stimulation A constant feature in these experiments was the partial recovery of the response which may occur even after the omission of 1—2 trains of stimuli suggesting that a certain degree of resynthesis was going on continuously (Fig 7) I v injections of noradrenaline (2—5 μg/kg) or dopamine (20—100 μg/kg) restore the vasoconstrictor response to a considerable degree over several stimulation periods The failure of dopa to restore the transmission effect is compatible with the report by Merritt and Schultz (1966) that decaborane inhibits the decarboxylation of dopa

Significance of free and bound NA in tissue homogenates

Since the early experiments in which splenic nerves were used as starting material for the preparation of granules it was observed that only a portion of the total NA content could be recovered in the granules while the remainder was present in the supernatant after high speed centrifugation With the squeezing method which has the advantage of yielding a relatively pure granule preparation the proportion of bound NA is fairly low about 30 per cent of the sum of granule bound and soluble NA Later experiments in which homogenization was achieved with the Ultra Turrax apparatus, yielded a rather higher proportion of particle bound NA up to 60 per cent

From the results of granules separation experiments as well as by observation on the distribution and release of radioactive NA *in vivo* and the effects of certain drugs on the stores it has been generally assumed that the transmitter is present in more than one pool (cf Axelrod 1965 There is so far no agreement however as to the nature of the extragranular pool or pools Most authors seem to agree that the amount of free occurring NA *in vivo* under normal conditions is small and this portion may be termed transport NA Euler 1966) After injections of NA a larger proportion may initially be found in the soluble portion and probably represents temporary flooding of the tissue with extragranular NA Wegmann and Kako 1961 It has been postulated that practically all of the NA in organs is normally granule bound and that the deficit observed in homogenates is due to damage to the granules during the homogenization process (Carlsson 1966) Recent experiments Euler and Lishajko 1966 c have shown that the granule bound portion diminishes when a suspension of granules is exposed to further treatment with the homogenizer Thus after 8 min at speed 13 300 rpm with the Ultra Turrax apparatus less than 10 per cent of the NA was found in the granular fraction Even treatment applied for the same time length as used for the original homogenization of the tissue caused a reduction of the granule bound by some 20 per cent The results of the homogenization experiments thus suggest that mechanical damage of granules causes release of the transmitter even at low temperatures at which spontaneous release is negligible He

results of sonication (Euler and Lishajko 1966 c) speak in the same direction One implication of these observations is that NA appears to be bound in such a way in broken up or damaged granules that it is susceptible to immediate release on exposure to some factors in the suspension medium, and that the intact granules offer protection against such factors

Since it cannot be excluded that the initial disintegration of the tissue during homogenization is fraught with damage to a certain proportion of the granules, evidence at present points towards the opinion that most of the NA in tissues normally is granule bound

Spontaneous NA release from granules may also contribute to a lowering of the proportion granule bound NA On leaving the rabbit heart for half an hour in the body after killing the animal, the proportion NA found in the high speed sediment from the heart, in relation to the sum of NA in the sediment and in the supernatant after separation of larger particles, is considerably decreased in comparison to the freshly excised heart (Euler and Lishajko 1966 c)

Thus if the interval between death of the animal and excision of organs is consider able as may be the case for bovine tissues a certain degree of release of granule bound NA is likely to occur This would be particularly noticeable in tissues in which granules show a fast release rate (splenic nerves, heart)

If the assumption is correct that most of the NA in nerves, and consequently in organs, supplied with such nerves is bound to granules then it might well be that the apparent proportions of bound and free NA in a tissue homogenate remain approxi mately the same whether the total amount is high or low Such a constancy of the distribution has in fact been observed in normal and decaborane-depleted rabbit hearts, even after refilling with injected NA (Euler and Lishajko 1965 b), and in analogous experiments with prenylamine (Mackenna 1965) and adrenaline (Westfall 1965) On the other hand it has been reported that after reserpine the proportion of granule bound and soluble NA is altered (Axelrod 1966) As mentioned above it cannot be excluded that spontaneous NA release in conjunction with disintegra tion of granules during homogenization may account for at least part of the free NA in homogenates

On the other hand various observations make it appear probable that some of the transmitted NA appears in a loosely bound transport form ready for immediate release upon nerve stimulation As postulated previously Euler 1966 the NA release from the granules and subsequent resynthesis may occur as a secondary phenomenon

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Studies of Noradrenaline Biosynthesis in Nerve Tissue

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Abstract

STJÄRNE L. *Studies of noradrenaline biosynthesis in nerve tissue.* Acta physiol. scand. 1966. 67. 441—454

Results recently obtained in studies of the *in vitro* synthesis of noradrenaline from tyrosine, dopa and dopam[illegible]

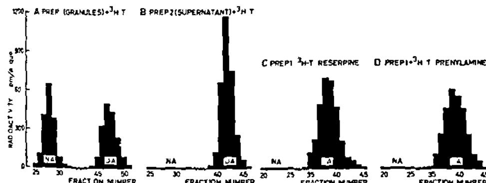
support the concept that deamination to a large extent occurs in the "granule fraction" which accord[illegible]

oxidase may attack only one of these "pools": the non bound intragranular amines while the amines in the remaining "pools" may be protected against this enzyme either by binding or by staying extragranularly. The results are summarized diagrammatically in a proposed model of the sympathetic neuro-effector junction.

The enzymes required for noradrenaline[1] (NA) biosynthesis from tyrosine (T) have been demonstrated in various tissues. However, the exact subcellular localization of the enzymes, and the mechanism whereby their activity is regulated, remains unknown or a matter of debate. The recent discovery of drugs specifically inhibiting individual steps in the synthetic sequence *in vivo* (Goldstein *et al.* 1964, Musacchio, Kopin and Snyder 1964, Spector, Sjoerdsma and Udenfriend 1965) and the evidence that certain pharmacologic agents known as NA "depleters" may exert part of their effect by blocking NA synthesis (Bertler, Hillarp and Rosengren 1961, Kirshner, Rorie and Kamin 1963), have emphasized the immediate practical relevance of clarification of this issue.

[1] Abbreviations: NA = Noradrenaline; DA = dopamine; dopa = dihydroxyphenylalanine; CA = catecholamines; T = tyrosine; MAO = monoamine oxidase.

The present paper, which is based on recent studies of the *in vitro* synthesis of NA from T dihydroxyphenylalanine (dopa) and dopamine (DA) in various fractions of the bovine splenic nerve homogenate (cf. Stjarne 1966), summarizes the findings concerning the subcellular localization of the different steps in NA synthesis (Stjarne and Lishajko 1966 a, Stjarne, Lishajko and Roth 1966 a), the regulation of synthesis (Stjarne, Lishajko and Roth 1966 b) and the drug induced inhibition of this process (Stjarne and Lishajko 1966 b). The conclusions drawn from the information obtained in these experiments have led to a concept for the functional organization of the noradrenergic nerve terminals which is presented diagrammatically in full realization of the need for future modification as new experimental facts emerge.



or prenylam ne 3×10^{-4}M (*D*) (From Stjärne and Lishajko 1966)

t me was 10 m n Quenching was mon tored by internal standards

Recovery The overall recovery of material passed through the entire procedure ranged between 70 and 90 per cent

Results and Discussion

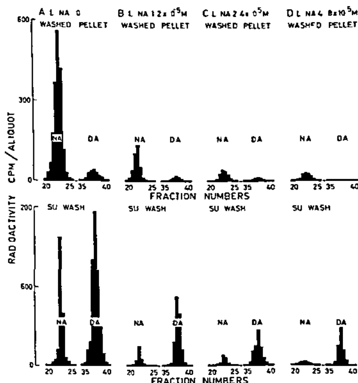
A *Subcellular localization of biosynthetic steps* The enzymes necessary for the local synthesis of NA from T have been demonstrated in sympathetic nerves (Goodall and Kirshner 1958) Until recently (Nagatsu Levitt and Udenfriend 1964) little was known about the enzyme catalyzing the ring hydroxylation of T but considerable information had been accumulated concerning the two enzymes involved in the decarboxylation of dopa (cf Holtz 1959 and the subsequent β hydroxylation of DA (cf Kaufman and Friedman 1965 Dopa decarboxylase has been reported to be located in the extragranular cytoplasm of the adrenal medulla (Blaschko *et al* 1955) while the DA β hydroxylase has been found in the specific CA containing granule fraction both from the adrenal medulla Kirshner 1957 and from the rat heart (Potter and Axelrod 1963) However it has recently been claimed that the T hydroxylase activity in the adrenal medulla brain and various peripheral sympathetically innervated tissues is particlebound Nagatsu *et al* 1964 McGeer *et al* 1965 and that indeed the whole of the biosynthetic sequence from T to NA can be demonstrated to proceed in this particulate fraction

In the present experiments the hydroxylation of T and the subsequent decarboxylation of dopa were found to proceed with equal eficiency in the particle f speed supernatant and in the particle containing low speed supernatant

and B) In the former case there was no synthesis of NA, but the amount of DA formed was about equal to the sum of the DA and NA formed in the latter medium Thus the granules were necessary for the conversion of DA to NA but not for the two previous steps (Stjarne and Lishajko 1966 a). On the other hand the yield of NA formed from T or dopa was very low, on incubation with granules isolated from their original medium and resuspended in potassium phosphate particularly after washing the granule fraction free from contamination with material from the super natant Similar results were obtained even after due fortification of the medium This makes it unlikely that any quantitatively important DA formation occurs in the granule fraction and rather supports the classical view that the first two steps in NA biosynthesis proceed extragranularly This is further supported by the finding that most of the DA formed in these experiments was recovered from the supernatant while usually more than 50 per cent of the NA formed was found in the washed sediment pellet Furthermore, drugs known to interfere with amine uptake in the granules like reserpine, prenylamine and phenoxybenzamine (Euler and Lishajko 1965, for adrenomedullary granules cf Carlsson et al 1963) were found to inhibit NA formation specifically at the DA β hydroxylation step the amounts of DA formed from T being equal to those found on incubation of the particle free high speed supernatant in the absence of drugs (Fig 1 C and D Stjarne and Lishajko 1966)

The discrepancy between the present results and those indicating that the T hydroxylase activity is particle bound might to some extent be due to organ and species differences However it appears more likely that it reflects differences in terminology The particulate fraction which was claimed by the abovementioned authors to contain the T hydroxylase activity consisted of the 15 20 000 g sediment apparently obtained from organ homogenates without previous removal of the coarse fraction (Nagatsu et al 1964) In the brain homogenates the enzyme activity was found in the synaptosome fraction McGeer et al 1965 Thus in both cases the "particulate fraction studied was very heterogenous consisting of cell membranes cytoplasm and intracellular organelles cf Gray and Whittaker 1962) The possibility that the T hydroxylase in these cases actually was confined to extra granular cytoplasm cannot be excluded and appears even more probable in view of the high enzyme content of the particle free supernatant Nagatsu et al 1964 Moreover, the only particulate fraction described which could be considered reasonably homogeneous and free from contamination with cytoplasm the 100 000 g sediment after removal of the 15 000 × g sediment was practically devoid of T hydroxylase activity Thus the available evidence seems to be best compatible with the concept that the first two steps in NA biosynthesis occur extragranularly while the last step proceeds in the granules

B *Regulation of NA biosynthesis* The hydroxylation of T which has been claimed to be the rate limiting step in NA biosynthesis has been shown to be significantly inhibited by a NA concentration in the medium of 2 10 $^{-4}$M suggesting possible feedback control of the initial step in NA synthesis Nagatsu et al 1964) In agree



F g 2 Feedback control of \A synthes s Ion exchange chromatograms of washed pellet and pooled supernatant and wash after incubat on of prep 1 w th labeled T at 20 C for 60 m n In this preparation the endogenous extragranular NA and DA were removed by alum na (see methods) pr or to the incubat on and l \ \ was added to final concentration of 0 12 24 or 48 nmol/ml (From Stjarne *et al* 1966 b)

ment with this an increase in the \A concentration in the medium in the present experiments strongly inhibited NA synthesis from T However in these cases a \A concentration of only 1 2 × 10 ⁵M was found to cause a marked and 2 4 × 10 ⁵M a more than 90 per cent inhibition of \A synthesis while D\ formation was much less affected This indicates that the synthesis block occurred not only at the first but to a large extent at the last step in the biosynthetic sequence (Stjarne *et al* 1966 b Fig 2) Thus it seems that \A in the medium can inhibit \A synthesis both by competitive inhibition of D\ uptake into the β hydroxylation sites and by product inhibition of thi and or any of the two previous enzymatic steps

This observation demonstrates the feasibility of the concept of \A synthesis acceleration by increased nervous activity and consequent partial depletion of some strategic \A pool in the tissues Euler 1962 Oliverio and Stjarne 1965 Roth Stjärne and Euler 1966)

Properties of the β hydroxylation mechanism and fate of ne ily formed \ 1 cf Stjarne *et al* 1966 a) Although the β hydroxylation of DA occurred exclusively in the granules, this process was found to proceed more efficiently in the low speed supernatant than

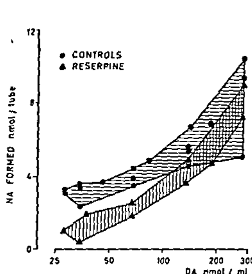


Fig 3

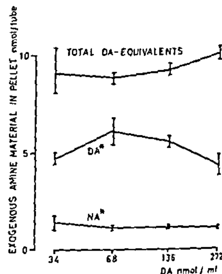


Fig 4

Fig 3 NA synthesis as a function of the DA concentration in the medium and competitive inhibition of synthesis by preincubation with reserpine. Prep 3 (resuspended granules) incubated with ^{3}H-DA and nonlabeled DA, with or without preincubation with reserpine 10^{-5}M (From Stjärne *et al* 1966 a)

Fig 4 Retention in the washed pellet of chromatographically identified DA (DA*) newly formed NA (NA*) and of total exogenous amine material calculated from total retention of radioactivity and expressed as DA equivalent, as a function of the DA concentration in the medium Prep 3 (resuspended granules) incubated with ^{3}H DA and nonlabeled DA (From Stjärne *et al* 1966 b)

in the resuspended granules, indicating the possible existence of cofactors in the original suspension medium, and presumably also reflecting the incompleteness of resuspension, making it more difficult for the DA to reach the β hydroxylation sites

The NA formation from DA increased with the DA concentration in the medium, in agreement with observations made in the isolated perfused guinea pig heart (Levitt *et al* 1965) At a level of 272 nmoles of DA per ml a rate of about 10 nmoles/g splenic nerve (wet weight)/hr was obtained (Fig 3) However the retention mechanism for the newly formed NA, as well as for DA, appeared to have a strictly limited maximum capacity of about 1 and 5 nmoles per washed pellet respectively, corresponding to about 10 and 50 per cent, respectively, of the total endogenous NA in the undepleted pellet (Fig 4) This implies that the newly formed NA did not mix with the endogenous NA, but was rather contained in a separate compartment of small capacity, located in the NA storage granules or possibly in separate "synthesis granules".

In order to define the exact localization of the store for newly formed NA, resuspended granules were incubated with constant amounts of labeled DA in the presence of increasing concentrations of nonlabeled l-NA, the molar ratio l NA/DA increasing to a maximum of 5,5 1 This resulted in a marked reduction in the

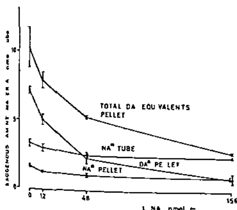


Fig 5

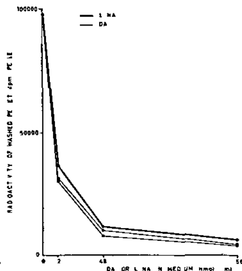


Fig 6

Fig 5 NA formation and retention in the washed pellet of DA (DA*) newly formed NA (NA*) and of total exogenous amine material expressed as DA equivalents as a function of the l NA concentration in the medium Prep 3 (resuspended granules) incubated with ³H DA 34 nmol/ml and l NA 0—156 nmol/ml (From Stjarne *et al* 1966 a)

Fig 6 Competitive inhibition of ³H dl NA uptake and retention in the washed pellet by l NA and DA in prep 3 (resuspended granules) (From Stjarne *et al* 1966 a)

amount of DA retained in the washed pellets, while NA formation, and the retention in the washed pellets of newly formed NA, were only moderately reduced (Fig 5) This implies that the uptake of DA into the β hydroxylation sites, even at the highest external NA concentration, although strongly reduced, was high enough to essentially maintain the NA formation at a level almost saturating the storage capacity for newly formed NA In these experiments the ratio of added l NA to newly formed NA reached a level of 700 1 The absence of profound reduction in the retention of the newly formed NA indicates that it was taken up by a retention mechanism immediately after formation from DA, without previous competition for uptake with the NA in the medium

Competition between DA and l NA for granule uptake and retention mechanisms Since the formation of NA from DA, on incubation of the low speed supernatant with T, was dependent on the NA concentration in the medium, it appeared of interest to determine the relative affinities of DA and l NA for the granule uptake and retention mechanisms Thus resuspended granules were incubated with a tracer dose of ³H dl NA together with increasing doses of nonlabeled l NA or DA, up to 156 nmoles/ml, and the uptake and retention of the labeled NA in the washed pellets was determined DA was found to competitively inhibit the tracer dl NA uptake and retention at least as efficiently as l NA (Fig 6) This finding seems to be in contrast to the report that the β hydroxyl group increased the "affinity" of various sympathomimetic

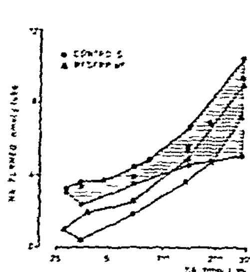


Fig. 3

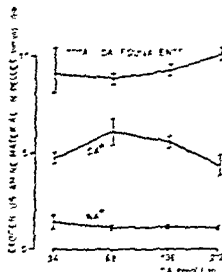


Fig. 4

Fig. 3. NA synthesis as a function of the DA concentration in the medium [illegible] Prep. 3, resuspended granules [illegible] ^{3}H-DA and unlabelled DA [illegible] From Stjärne *et al.* [illegible]

Fig. 4. [illegible] newly formed NA [illegible] and expressed as DA equivalents as a function of the DA concentration in the medium. Prep. 3, resuspended granules incubated with ^{3}H-DA and unlabelled DA. From Stjärne *et al.* [illegible]

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The NA formation from DA increased with the DA concentration in the medium in agreement with observations made in the isolated perfused [illegible] (e.g. Levin *et al.* 1965). At a level of 272 nmoles of DA per ml a [illegible] of 10 nmoles/g splenic nerve wet weight [illegible] was obtained (Fig. 3). However, [illegible] mechanism for the newly formed NA, as well as for DA, appeared to have a strictly limited maximum capacity of about 1 and 5 nmoles per washed pellet, corresponding to about 10 and 50 per cent, respectively, of the total endogenous NA in the undepleted pellet (Fig. 4). This implies that the newly formed NA did not mix with the endogenous NA but was rather contained in a separate compartment of small capacity, located in the NA storage granules or possibly in separate "immature granules".

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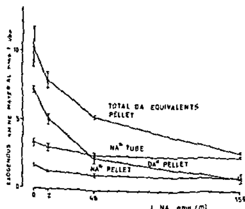


Fig 5

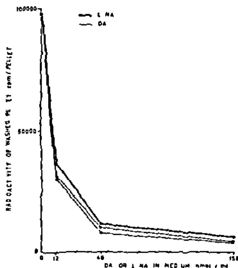


Fig 6

Fig 5 NA formation, and retention in the washed pellet of DA (DA*) newly formed NA (NA*) and of

amount of DA retained in the washed pellets, while NA formation, and the retention in the washed pellets of newly formed NA, were only moderately reduced (Fig 5) This implies that the uptake of DA into the β-hydroxylation sites, even at the highest external NA concentration, although strongly reduced, was high enough to essentially maintain the NA formation at a level almost saturating the storage capacity for newly formed NA In these experiments the ratio of added l-NA to newly formed NA reached a level of 700 1 The absence of profound reduction in the retention of the newly formed NA indicates that it was taken up by a retention mechanism immediately after formation from DA, without previous competition for uptake with the NA in the medium

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amines for uptake and retention in the NA storage granules of the rat heart (Musacchio, Kopin and Weise 1965).

Drug effects on NA synthesis in vitro. Several drugs were found to inhibit NA synthesis *in vitro* in fractions from the splenic nerve homogenate (Stjarne 1966, Stjarne and Lishajko 1966 b). Thus, as previously mentioned, reserpine and prenylamine completely inhibited NA formation from DA in the low speed supernatant, while not affecting DA formation (Fig. 1, C and D). When resuspended granules were used it was observed that preincubation with the drug was required in order to obtain a maximum inhibitory effect on NA synthesis, and on the uptake of the newly synthesized NA into a wash resistant storage mechanism. However, even after preincubation of the resuspended granules with the drug, the synthesis mechanism was found to be much less sensitive to the inhibitory effect of reserpine than the mechanism for retention of DA and newly synthesized NA. In both cases the inhibitory effect of reserpine could be to a large extent overcome by raising the DA concentration in the medium (Fig. 3, Stjarne *et al.* 1966 a).

The present results provide experimental support for the hypothesis that reserpine affects amine transport at more than one level in sympathetically innervated tissues (Stjarne 1964). They also show that the inhibitory effects of this drug on synthesis of NA, and on uptake and retention of the newly formed NA, are of a competitive nature, in agreement with previous findings for the inhibition of exogenous NA uptake in CA-containing granules (Carlsson *et al.* 1963, Stjarne 1964).

The observed difference in sensitivity to the inhibitory effect of reserpine on the synthesis and binding mechanisms may have important clinical implications. Thus it appears that after reserpine administration the synthesis of NA may recover at a time when the continued binding block prevents an accumulation of NA in the store. It is conceivable that this restoration of synthesis capacity may be even more closely related to the return of function than the restoration of tissue uptake of circulating NA (Andén *et al.* 1964, Stjarne 1964). This may thus be the cause of the rapid disappearance and gradual reappearance of function, accompanying the waxing and waning drug concentration following each new reserpine injection in the chronically reserpine treated and therefore essentially NA-depleted rabbit (Häggendal and Lindqvist 1963).

However, the drug experiments also provide the important information that synthesis and binding involve two different transport steps, one responsible for DA transport to the β-hydroxylating enzyme and the second for carrying the newly formed NA into its ultimate storage "compartment". As previously mentioned the absence of competitive inhibition of the retention of newly formed NA by addition of large amounts of NA to the medium implies that the second step must be located in the same particle as the first.

The "wash fraction". Washing the high speed sediment pellet obtained on centrifugation of the resuspended granule preparation, after the end of the incubation period, resulted in removal from the pellet of a considerable amount of amine material (DA and/or metabolites), here called the "wash fraction" (cf. Stjarne *et al.*

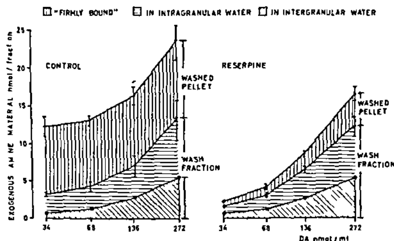


Fig 7 Exogenous amine material in the washed pellet (firmly bound) and in the wash fraction (20 [illegible]

1966 a) This fraction was found to be less easily saturable than the wash resistant fraction" (above called washed pellet), and turned out to accomodate large amounts of the material originally added to the incubation medium (Fig 7) When low DA concentrations were present in the medium the amine material found in the wash fraction corresponded to the value expected, provided DA was able to penetrate throughout the simultaneously determined tritiated water space However, when larger DA concentrations were used or when increasing amounts of l NA were added to the medium there was a progressive decrease in the relative amount of amine material in the wash fraction Preincubation with reserpine also depressed the yield of amine material in the wash fraction, at low DA concentrations in the medium but not at higher DA concentrations

The tritiated water space determined in the present experiments consisted of intragranular water intergranular water and water contaminating the walls of the tube and the surface of the pellet The results show that DA could penetrate throughout this space, and therefore also through the granule m brane into the intragranular water This is in agreement with previous findings in the adrenomedullary (Carlsson and Hillarp 1958 Hillarp 1959) as well as splenic nerve granules (Stjarne 1964) leading to the conclusion that the granule membrane is permeable to CA However, according to the present experiments the permeability to CA of the nerve granule membrane is not completely free Although DA at low concentrations was equally distributed on either side of the membrane, the passage across the membrane of tracer amounts of DA or dl NA was competitively inhibited by larger concentrations of DA or l NA, and also by preincubation with reserpine This suggests that amine penetration into the intragranular water space occurs by diffu

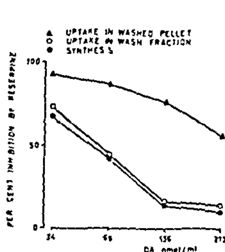


Fig 8

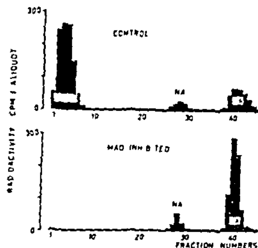


Fig 9

Fig 8 Inhibitory effect of reserpine on uptake and retention in the washed pellet and in the wash fraction and on β hydroxylation (DA synthesis as a function of the DA concentration in the medium Calculation based on the assumption that 20 μl of the total tritiated water space represents trapped intergranular water cf text to Fig 7 Prep 3 resuspended granules incubated with ³H DA and nonlabeled DA with or without reserpine 10 ⁵M From Stjärne et al 1966 a)
Fig 9 Effect of MAO inhibition on the nature of the wash fraction Ion exchange chromatograms of the wash fraction after incubation of prep 3 (resuspended granules) with ³H DA and nonlabeled DA (final DA concentration 34 nmol ml with or without preincubation with the MAO inhibitor pargyline 10 ⁴M (From Stjarne et al 1966 a)

through a limited number of "pores" in the granule membrane and that CA passage through these "pores" can be to some extent prevented by reserpine The maximum inhibitory effect of reserpine appears to occur at a molar amine reserpine ratio of about 1 1 At a ratio of 13 6 1 the drug was practically devoid of inhibitory effect on the penetration of amines through the granule membrane

Preincubation with reserpine decreased the amount of amine material in the wash fraction to about the same extent as it inhibited NA synthesis from DA Fig 8) Thus it appears that DA must penetrate through the granule membrane in order to get access to the β hydroxylating enzyme which might be located on some structure inside the granules (Kirshner 1962) possibly on the interior surface of the granule membrane This could then imply that the granule membrane itself might contain the low-capacity storage mechanism for newly synthesized NA, postulated in the present study

Effects of MAO inhibition In the present experiments MAO inhibition did not appreciably affect NA formation, either from T or from DA, in agreement with the *in vivo* observations of Levitt and coworkers (1965) This appears to imply that DA was able to reach the β hydroxylation sites without being exposed to MAO

MAO inhibition did not affect the size of the wash fraction However, it strikingly altered the nature of the material retained in this fraction Chromatographic analysis revealed that the wash fraction, in the absence of MAO inhibition, to a large extent



Fig 10 Electron micrograph (Euler and Forsling personal communication) of the high speed sediment obtained from bovine splenic nerve by centrifugation of the press juice (*cf* Euler 1958) at 50 000 × *g* for 30 min, after previous removal of the coarse fraction by centrifugation at 9 000 × *g* for 10 min Magnification × 13 000 This sediment was found to contain the bulk of the MAO-activity in the whole homogenate (From Roth and Stjarne 1966)

consisted of deaminated material, occurring at a considerably higher concentration than in the supernatant, while after MAO inhibition essentially the same amount of material was recovered as intact DA and traces of newly formed NA (Fig 9) This suggests that deamination actually occurs in the wash fraction, and thus possibly in the NA-storing granules themselves

According to recent evidence (Roth and Stjarne 1966) the MAO activity in splenic nerve tissue is to a large extent contained in a sedimentable fraction, which according to electron microscopic evidence is morphologically relatively homogenous and apparently largely free from contamination with mitochondria (Fig 10) The present results actually suggest that at least part of the MAO in the axon may be located inside the membrane of the NA storing granules This would explain why NA formation from DA, *in vivo* and *in vitro*, is not affected by MAO inhibition, since according to the above mentioned proposition the β-hydroxylating enzyme may be located in this membrane If this concept is correct it appears to lead to the important consequence that amines, in sympathetically innervated tissues *in vivo*, can be protected from destruction by MAO in two ways either by being contained in the specific amine binding mechanism in the granule substructure' (*cf* Stjarne 1964), or by staying outside the granules in the extragranular axoplasm ("free NA pool")

Conclusions

The results obtained in the present study seem to warrant the following conclusions

1 The hydroxylation of T and the subsequent decarboxylation of dopa, in bovine splenic nerve tissue take place in the extra granular axoplasm, while the formation of NA from DA occurs in the granules

2 The synthesis of NA is regulated by the NA concentration in the tissues in two ways By product inhibition of the enzymatic processes themselves, probabl essentially at the first step in the sequence, the hydroxylation of T, and by competi inhibition of DA binding to the β-hydroxylation sites

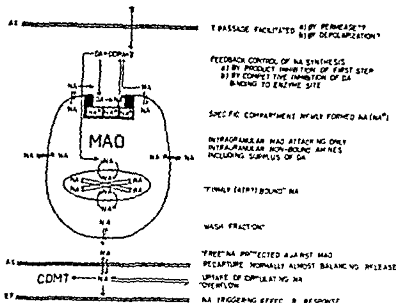


Fig 11 Diagram proposed to represent some of the basic features of the noradrenergic neuro-effector junction

better substrate than NA d Newly synthesized NA kept in a separate compartment (in the granule membrane? which has a strictly limited storage capacity the surplus passing out into the intra granular and or extragranular water

2 *Locali at* [illegible] *MAO* [illegible] *MAO* [illegible]
most of the [illegible]
possibly in th [illegible]
be protected [illegible]
the granules

3 NA "pools a Firmly bound NA in granule substructure b NA dissolved in granule water c Specific store for newly formed NA d Free extragranular NA Granule bound (?) MAO attacks only pool b dissolved in granule water?

3 The β hydroxylating enzyme is 'non saturable while the storage capacity for newly formed NA has a strictly limited capacity, corresponding to less than 10 per cent of the total NA in the non-depleted granules Thus the storage system for the newly synthesized NA constitutes a well defined separate NA pool in the granule fraction This NA compartment is situated in immediate proximity to the β hydroxylation site

4 DA inhibits tracer d l NA uptake and retention in the granules at least as efficiently as l NA

5 The permeability to CA of the granule membrane is limited, and appears to be

inhibit tracer amine penetration into the granule water. Reserpine also competitively inhibits amine penetration through the postulated pores, the maximum inhibitory effect occurring at an amine/reserpine ratio of about 1 : 1. At a ratio of 13.6 : 1 or higher reserpine cannot prevent amine passage through this membrane.

6. DA must penetrate into the intragranular water space in order to get access to the β-hydroxylating enzyme, which seems to be located inside the granules, possibly on the interior aspect of the granule membrane.

7. MAO inhibition does not appreciably affect *in vitro* NA synthesis from T or DA in fractions from the splenic nerve homogenate. This may be explained by assuming that DA reaches the β-hydroxylating enzyme before it is exposed to MAO. The results are compatible with the view that MAO is actually contained in the NA storing granules themselves, inside the granule membrane, in which the DA β-hydroxylase appears to be located. This assumption is supported by the following observations:

a. The major part of the MAO in this tissue was obtained from particles morphologically resembling the NA storage granules.

b. Deaminated material was found to be concentrated in this sedimentable fraction.

c. On MAO inhibition corresponding amounts of intact amines were found in this MAO exposed part of the granule fraction.

These conclusions are summarized diagrammatically in a proposed model of the sympathetic nerve endings (Fig. 11).

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From the Department of Pharmacology, Karolinska Institutet Stockholm, Sweden

Mode of Storage of Histamine in Mast Cells

By

I L Thon and B Uvnäs

Abstract

Thon, I L and B Uvnäs *Mode of storage of histamine in mast cells* Acta physiol scand 1966 67 455—470

Histamine containing granular material was collected from water lysed peritoneal rat mast cells The histamine was observed to be released in the presence of cations A re uptake of the amine occurred on suspension of depleted granular material in histamine containing solutions Release as well as uptake of histamine occurred rapidly at 0° C. The results are in accordance with the idea that histamine is electrostatically bound to a granular protein heparin complex the release and uptake of the amine being a physical process depending on the extra and intra granular concentrations of histamine and cations

Mast cells from rats contain 10—15 μg histamine (free base) per million cells (Benditt *et al* 1956, Uvnas 1964) The histamine concentration in the granules is high, 0 6 M (Uvnas 1964)

The concomitant occurrence of histamine and heparin in the mast cells has aroused speculations as to an ionic linkage between the amine and the acid polymer (McInture 1956, Paton 1956, Green and Day 1960, Green 1962 Uvnas 1963, 1965, Benditt and Lagunoff 1964) The experimental evidence presented to support this hypothesis, however, has been somewhat poor, the argumentation resting mainly on two observations 1) The ability of heparin to form *in vitro* a heparin-histamine complex, which can be broken with cations (Keller 1958 Kuttner 1961) 2) The ease with which histamine can be released from tissues by ice-cold trichloracetic acid, alcohol, acetone, distilled water and other agents incapable of breaking covalent chemical bonds (Threthewie 1938 McInture Roth and Shaw 1947)

However, the ability of distilled water (with or without repeated freezing and thawing of the material) and of lytic substances to release practically all the histamine from particles isolated from liver and mast cell homogenates has been taken to indicate that the histamine is enclosed within a membraneous particle Thus, the histamine would exist in a diffusible form and could escape on rupture or on increased permeability of the limiting membrane (Grossberg and Garcia-Arr 1954, Garcia Arocha 1961)

Several investigators have isolated histamine-carrying particles from liver and mast cell homogenates. These particles have retained histamine even on suspension in Tyrode's solution (Garcia-Arocha 1961) or other isotonic salt solutions (Hagen 1954). These results are difficult to reconcile with the existence of a heparin-histamine ionic linkage, unless the granules are surrounded by a membrane impermeable to cations. However, according to recent electron-microscopic studies, mast cell granules from hamster and rat lack a definitive membrane, (Fernando and Movat 1963, Benditt and Lagunoff 1964, Bloom and Haegermark 1965).

The present experiments were designed to elucidate the mode of storage of histamine in mast cells and to explain the above-mentioned discrepancies between the results obtained by previous investigators. Preliminary reports have been given at several symposia (Uvnäs 1963, 1964, 1965).

Methods

Isolation of mast cells

Male Sprague-Dawley rats weighing 400–500 g were anesthetized with ether and bled by cutting the carotids. Nine ml of isotonic sodium chloride solution (adjusted to pH 6 with 10 per cent Sørensen buffer) were injected into the rat abdomen. After careful massage for one and a half min the abdomen was opened in the midline, the intestines were moved aside, and the abdominal fluid was sucked off with a drop pipette. In a similar way, the pelural cavities were flushed with saline through an opening made in the diaphragm just below the xiphoid process.

Mast cells were isolated from the wash fluid by differential centrifugation in Ficoll as described previously (Uvnäs and Thon 1961) with the minor modification that only two layers of Ficoll (30 and 40 per cent by weight) were used. Before experiments were performed in ion poor medium the mast cells were washed once in isotonic sucrose solution at 0° C following the regular washing in salt albumin solutions. Care was taken not to prolong unnecessarily the exposure of the mast

Isolation of granular material

Mast cells were disrupted either by suspension in distilled water for 5 min at room temperature or by repeated freezing and thawing (at least 3 times) in isotonic sucrose solution. By centrifugation of the water lysed cells (20 min at 90 × *g* and at 2 700 × *g*) two sediments were obtained. The first sediment contained clusters of granules adhering to cell fragments, the second contained "free" granules. Due to the strong tendency of the granules from water lysed cells to adhere to cell fragments and form heavy clusters sedimenting at 90 × *g*, the yield of "free" granules was low. However when the mast cells were disrupted in isotonic sucrose solution (pH 7) or when water lysed cell

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solution (see Fig. 4). The release could be

n in Tyrode's solution with continuous

base

al was boiled for 5 min in isotonic saline

procedure to be 100 per cent effective

is given 4 mC ^{35}S (Na_2SO_4) s.c. 2–3 days

previously

TABLE I Partition of "free" and particle bound histamine and heparin after physical disruption of mast cells, 5×10^{5}/ml, in various media

Procedure	Histamine per cent		Heparin per cent	
	Supernatant	Sediment	Supernatant	Sediment
Freezing and thawing twice in distilled water	30	70	4	96
Freezing and thawing 3 times in isotonic sucrose	26	74	2	98
	31	69	3	97
Freezing and thawing 3 times in isotonic NaCl	100	0	56	44
	100	0	62	38
Lysis in distilled water 20°C, 5 min	24	76	4	96
	28	72	6	94
Lysis in distilled water 20°C subsequent sucrose to isotonicity	34	66	12	88
	39	61	9	91
After lysis in distilled water isotonic NaCl to the sediment	100	0	0	100

Ficoll was dissolved in 0 9 % *sodium chloride solution buffered to pH 7 with 10 per cent Sorensen* phosphate buffer After 2 hrs at 60° C 1 % albumin and 2 % glucose were added

³⁵S-activity was recorded with a Tri Carb liquid scintillation counter Packard Instrument Company Inc, La Grange, Ill USA

Isotonic sucrose 0 28—0 31 M in distilled water

Isotonic sucrose pH 6 9 isotonic sucrose was adjusted with KOH (KOH 10^{-4} M) to pH 6 9

Isotonic salt solution pH 6 9 0 15 M NaCl $2\ 7 \times 10^{-3}$ M KCl 10^{-3} M CaCl 10 % Sorensen phosphate buffer, 1 per cent human serum albumin

Ficoll mol wt 370 000 Pharmacia Co Uppsala Sweden

^{35}S (Na_2SO_4) Norwegian Institute Atomic Energy Kjeller Lillestrom Norway

Results

Partition of heparin and histamine on disruption of mast cells by freezing and or osmotic lysis

Mast cells containing ^{35}S heparin were suspended in distilled water or in isotonic sodium chloride solution After repeated freezing and thawing the material was centrifuged at 0° C for 15-20 min at 2 700 × g Other samples of mast cells were lysed by suspension in distilled water at room temperature for 5 min, with or without the subsequent addition of sucrose or sodium chloride to isotonicity The cell material was then centrifuged at 2 700 × g for 15—20 min

Table I summarizes the results Practically all ^{35}S activity was recovered from the sediments with one exception When the mast cells were frozen in isotonic saline, more than 50 per cent of the ^{35}S activity appeared in the supernatant

The low solubility of the ^{35}S-material was further demonstrated during the incubation of the sediment from water lysed mast cells for 4 hrs at 37° C in distilled water, or in isotonic saline, when the supernatant ^{35}S activity rose only to 15 per c. and 11 per cent respectively (Table II)

TABLE II. Influence of time on release of histamine and heparin (^{35}S-activity) from water lysed mast cells 6×10^4/ml. Sediment $2\,700 \times g$ resuspended in water or in isotonic NaCl 37° C.

	Per cent histamine in supernatant			
	Water		Saline	
Hours	Histamine	Heparin	Histamine	Heparin
—	18	0	100	0
1	22	4	100	3
2	24	8	100	7
4	31	15	100	11

TABLE III. Influence of suspension volume on the release of histamine. Mast cells frozen 3 times in isotonic sucrose or in distilled water (1 ml). After dilution the cell debris was incubated for 15 min at 0° C and centrifuged at $2\,700 \times g$.

	Histamine per cent in supernatant	
Mast cells per ml	Sucrose	Distilled water
16×10^4	17	17
16×10^4	22	23
8×10^4	27	27
4×10^4	31	33
2×10^4	38	37
10^4	59	59

TABLE IV. Release of histamine on lysis of mast cells 3×10^4/ml in distilled water. Material resuspended in A water, B isotonic sucrose at 20° C.

	Histamine per cent in supernatant	
	A	B
Lysis	37	36
Resuspension 1	13	10
Resuspension 2	8	4

The distribution of heparin and histamine from disrupted cells did not run parallel i
sucrose,
natant histamine fraction increased with the suspension volume (Table III).
contrast, all histamine passed over to the supernatant fraction in isotonic saline

TABLE V Release of histamine on resuspension of sediment ($2\,700 \times g$) from water lysed mast cells 2×10^{5}/ml in water or sucrose

Incubation medium	Per cent histamine in supernatant after incubation at 37°C		
	5 min	30 min	60 min
Distilled water	24%	25%	29%
Sucrose pH 5 7	14%	15%	18%
Sucrose pH 7	5%	8%	9%

TABLE VI Influence of time and temperature on release of histamine from sediments ($2{,}700 \times g$) resuspended in sucrose pH 7 Mast cells 3×10^{5}/ml lysed by freezing 3 times in sucrose

Time of incubation	Per cent histamine in supernatant		
	0°C	37°C	50°C
5 min	10%	12%	13%
30 min	12%	11°	20%
60 min	11%	10°	19%

The sediments could be repeatedly resuspended in the same volume with only relatively small losses of histamine (Table IV) However, as a whole, greater changes in osmolarity, temperature, etc were deleterious to the storage mechanism, resulting in a considerable "leakage" of histamine from the sediments

Table V illustrates the fact that the loss of histamine from suspended particles occurs immediately Sediments ($2\,700 \times g$) from lysed cells were suspended at 37° C in distilled water, or in isotonic sucrose solutions of pH 5 7 and 7 (pH adjusted with KOH) After an initial histamine release no further appreciable "leakage" was observed during the following 60 min

The ability of the sediments to retain their histamine within the temperature range 0°—50° C is shown in Table VI After the initial loss of histamine on resuspension of the sediments the subsequent loss during a 1 hr incubation period was less than 10 per cent even at a temperature of 50° C

Isolation of histamine carrying granules by differential centrifugation

As evident from Table I and II the granular heparin was only slightly soluble in water or in saline thus offering opportunities for the isolation of the storage particles and the study of their storage mechanism

The centrifugation of water lysed mast cells at $90 \times g$ and at $2\,700 \times g$ yielded three ^{35}S and histamine carrying fractions Fig 1 After staining the heavier sediment with toluidine blue and examining it under the microscope it was obser

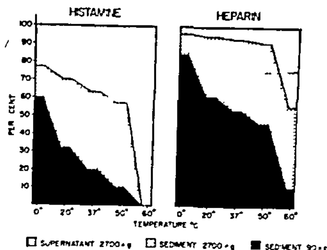


Fig 1 Influence of temperature on partition of histamine and heparin (^{35}S activity) from water lysed mast cells 5 · 10^6/ml
Note Histamine was totally released from sediments 90 × *g* and 2 700 × *g* on addition of NaCl to isotonicity

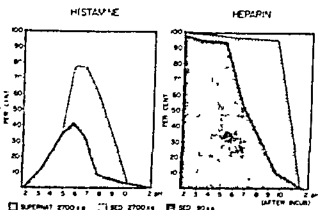


Fig 2 Influence of pH and cation concentration on partition of histamine and heparin (^{35}S activity) Mast cells 5 × 10^6/ml lysed in water of various pH at 20° C for 5 min pH adjusted with HCl and KOH
Note Histamine was totally released from sediments 90 × *g* and 2 700 × *g* on addition of NaCl to isotonicity The pH figures represent values after lysis

to contain large numbers of metachromatic granules, which formed clusters adhering to cell fragments The lighter sediment held numerous "free" metachromatic granules Only a minor fraction of the ^{35}S-activity (usually 5 per cent or less) was recorded in the supernatant after 2 700 × *g*

On water lysed mast cells the temperature was observed to influence the distribution of heparin and histamine after differential centrifugation (Fig 1) At 0° C the "free" granular fraction held only 10—15 per cent, and the supernatant fraction less than 5 per cent of the total ^{35}S-activity With rising suspension temperature up to about 50 C, the "free" granular fraction increased at the expense of the heavy sediment Above this temperature the solubility of the heparin rose, with more ^{35}S-activity appearing in the supernatant

The distribution of histamine from water lysed mast cells showed a similar dependence on temperature *although quantitatively it was slightly different* At 0° C about 25 per cent of the histamine was recovered in the supernatant (*cf* Table I) only 10—20 per cent being found in the "free" granular fraction The supernatant

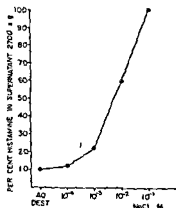


Fig 3

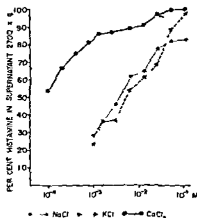


Fig 4

Fig 3 Release with NaCl of histamine from total sediment (2 700 × g) of water lysed mast cells 6 × 10⁵/ml, 0° C 5 min

Fig 4 Release with cations of histamine from granules isolated from mast cells 6 × 10⁵/ml disrupted by freezing in isotonic sucrose Granules resuspended at 0° C for 15 min in isotonic sucrose salt mixtures

and particularly the 'free' granular fraction, progressively increased with rising temperature until at around 60° C all the histamine went into solution

The pH influenced the distribution of ³⁵S-activity (Fig 2) Below pH 4 5, practically all ³⁵S-activity remained in the heavy (90 × g) sediment and only a few "free" granules were observed As the pH increased, a shift occurred from the heavy sediment to the 'free" granular fraction, which at pH 9—10 held about 90 per cent of the total ³⁵S-activity Above pH 10 the ³⁵S-activity passed over into the supernatant, indicating a dissolution of the heparin in this alkaline milieu

The granular-bound histamine showed optimal values around pH 7, a favourable pH range for harvesting histamine carrying "free" granules

The ³⁵S-activity and the histamine found in the supernatant after 2 700 × g remained in this fraction even after centrifugation at 100,000 × g

Release of histamine with ions

The observed ability of the granule-containing sediments to retain their histamine on suspension in water, and the breakdown of the binding mechanism in isotonic saline (tables and Fig 1 and 2) prompted a series of experiments on the release of histamine in the presence of ions

Cations

Fig 3 shows the histamine release from the total sediment (2 700 × g) of w[illegible] lysed mast cells on suspension of the sediment in unbuffered sodium chloride [illegible]tion (pH around 6, 0 C) With the amount of cell material used (6 × [illegible] the release of [illegible]

TABLE VII *Release of histamine from sediments (2,700 × g) of water lysed mast cells 6 × 10⁴/ml, suspended in serum and other isotonic cation containing solutions 0 C for 5 min*

Incubation medium	Histamine released %
Fresh rat serum	100
Tyrode's solution	100
Isotonic NaCl	100
Isotonic K phosphate buffer pH 7	100
Distilled water	8

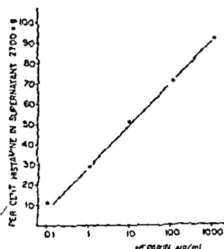


Fig 5 Release with heparin of histamine from sediments 2 700 g of mast cells 2 10⁴/ml disrupted by freezing in isotonic sucrose Sediments resuspended in isotonic sucrose pH 7 containing heparin at 37° C for 30 min

M and all histamine has passed over to the supernatant at a sodium chloride concentration of 10⁻¹ M To exclude the possible influences of osmotic variations, and of the admixture of nongranular cell debris on the histamine release sucrose isolated granular material was exposed to cations in isotonic salt sucrose mixtures No qualitative differences were observed between the releasing effects of sodium, potassium and calcium chloride (Fig 4) The tonicity of the suspension medium was evidently of no significant importance, even though a slight inhibition of sodium chloride-induced release was discernible in isotonic sucrose medium (80 per cent release in Fig 4 against total release in Fig 3)

Table VII shows the total release of histamine from sediments from water lysed mast cells (2 700 × g) suspended in serum, and in various isotonic salt solutions No significant release of heparin occurred in any of the above experiments

Comments

No qualitative difference was observed between the cation induced release of histamine from two granular containing sediments used (the heavy granular containing material with admixture of coarse cell debris, and the lighter "free" granule" fraction) Sodium, potassium and calcium were all active as releasers

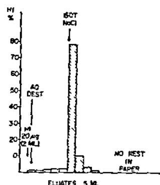


Fig 6 Retention of histamine (20 µg hi dihydrochloride) in filter paper Ederol No 14 and subsequent release with isotonic sodium chloride solution

Anions

Heparin If histamine is electrostatically bound to the granular matter, the balance within the granular histamine complex should be disturbed in the presence of an extragranular acid *polymer capable of complex binding with histamine* As evident from Fig 5, the addition of heparin to a granular sediment isolated in isotonic sucrose solution (pH 7 at 2 700 × *g*) caused a shift of histamine from the granules out into the suspension medium Identical results were obtained with granular material suspended in water No significant release of heparin occurred

Comments

The heparin used was a sodium salt containing about 100 mg Na/g and about 140 units of heparin/mg The mol wt of the heparin was claimed to be 7 000—12,000 In the above experiments 100—500 µg heparin ml were used, corresponding to a sodium concentration around 10^{-3} M Therefore, the shift of histamine to the suspension fluid could be due only to a minor degree to the presence of sodium ions (see *e g* Fig 3 and 4)

Cellulose When a histamine solution (in water or sucrose) is allowed to pass through a filter paper the amine is retained in the paper The histamine is released on washing the paper with a salt solution *e g* sodium chloride solution Fig 6 illustrates the total retention by a filter paper (Ederol No 14) of 20 µg histamine dihydrochloride dissolved in 2 ml of distilled water No histamine appeared in the effluent in spite of subsequent thorough washing with distilled water (over 20 ml) All the histamine was rapidly eluted shifting to perfusion with isotonic saline (NaCl) Twenty µg histamine dihydrochloride correspond to the amount found in 10^6 mast cells as used below

Ederol No 14 *is a rather fine pore* (mesh) filter paper When a suspension of disrupted mast cells in distilled water or sucrose is poured onto such a paper most of the microscopically visible cellular matter is withheld However, part of the granules pass through On centrifugation at 2 700 × *g* these form a homogenous sed of metachromatic granules without contamination with nuclei or other visible granular structures

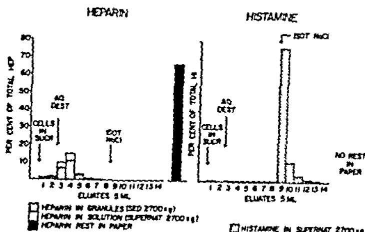


Fig. 7. Retention in filter paper Ederol No. 14 of histamine (total) and heparin containing granules (partial) from mast cells 10^6.
Note The granules were histamine free. The retained histamine was totally released from the filter paper on perfusion with isotonic sodium chloride solution.

In the experiment illustrated in Fig. 7, 10^6 mast cells containing ^{35}S labelled heparin were disrupted on filter paper by perfusion with 30 ml distilled water. The suspension was poured onto a filter paper (Ederol No 14) and the paper was subsequently washed with distilled water (30 ml). No histamine was eluted, but about 30 per cent of the radioactivity passed out with the effluent. After centrifugation at $2\,700 \times g$ for 20 min, the sediment showed metachromatic granules as described above. On shifting the perfusion fluid from water to isotonic saline all histamine passed out into the eluate.

Comments

The mechanism by which histamine is retained by filter paper is not clear. However, whether the retention is due to the presence of acid groups in the cellulose or to other attractive forces, the appearance of histamine-free granules in the perfusate from disrupted mast cells indicates a weak electrostatic binding of the cellular histamine to the granular protein-heparin complex.

Whatever the release mechanism may be, the main achievement of the perfusion experiment was the yield of histamine-free granules suitable for studies on the uptake and the secondary release of histamine (see page 00).

Rate of histamine release

The technique used did not allow any exact observations as to the rate of the histamine release since centrifugations and other procedures required at least 25 min, during which time an uncontrollable contact between the granular material and the ion-containing medium was unavoidable. It was observed, however, that all the histamine was released even when all procedures were performed at 0° C.

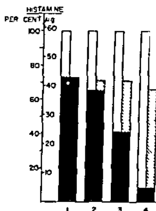


Fig 8 Re uptake of histamine into total sediments (2,700 × g) from water lysed mast cells (5 × 10⁶ cells per sample) deprived of their original histamine by treatment for 5 min at 20° C with heparin

1 Distilled water, 2 Heparin 20 µg/ml, 3 Heparin 100 µg/ml 4 Heparin 500 µg/ml

Incubation with histamine dihydrochloride 150 µg/ml 20 min 20 C pH 5

Note Re-uptake of histamine to the same level irrespective of the heparin concentration and degree of depletion

Histamine content in sediments

□ initial

■ rest after heparin treatment

□ re uptake on incubation with histamine

centrifugation was started immediately after the suspension of the granular matter in isotonic saline

The rapidity of the cation-induced histamine release was evident from the behavior of the guinea pig ileum on exposure to granular-bound histamine No differences could be observed between the latencies and the time courses of the contraction responses to histamine added as a suspension of histamine-containing granules or as a corresponding amount of dissolved histamine

Uptake of histamine into granules

Sediments (2 700 × g) from water lysed mast cells were deprived of their histamine by suspension in heparin solutions of various concentrations (Fig 8) The highest heparin concentration caused a reduction of the histamine content to less than 10 per cent of the initial level After resuspension of the sediments in histamine dihydrochloride (150 µg/ml), followed by washing in distilled water, the histamine content was found to have risen in all the heparin-treated sediments to about the same level as in the non-treated sample

Comments

It might be argued that heparin-treated granules do not represent a normal granular material since the uptake of extragranular heparin into the granules might influence their histamine binding capacity The fact that the histamine uptake reached the same level (= the initial level) independently of the external heparin concentration argues against such an explanation However to avoid the possible influence of external heparin on the storage capacity observations were also made on granules isolated by filtration

Mast cells (6 × 10⁶) were lysed in distilled water and granules were isolated and deprived of their histamine by filtering through Ederol No 11 filter paper as described above The granules, divided into 7 samples were incubated at various pH in histamine dihydrochloride 5 · 10⁻⁴ M for 15 min at 20 C

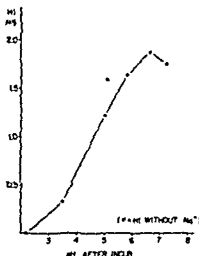


Fig. 9

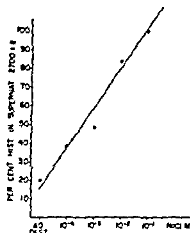


Fig. 10

Fig. 9. Influence of pH on uptake of histamine into histamine depleted granules isolated with filter paper Ederol No. 14.
According to toluidine blue titration 10—15 µg heparin per sample. 10^6 mast cells contain about 70 µg heparin; pH adjusted with HCl, phosphate buffer and NaCl. Sodium concentration 10^{-3} M throughout.
Fig. 10. Release with sodium chloride of histamine from granules depleted of histamine by passage through filter paper Ederol No. 14 and refilled by incubation in histamine solution 5×10^{-4} M, pH 6.5, 20° C, 5 min. 2.7 µg histamine per granule sample.

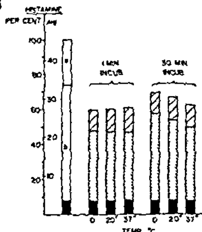


Fig. 11. Influence of time and temperature on re-uptake of histamine into heparin treated granules.
"Free granules" from water lysed cells 4×10^6 per sample treated with heparin 500 µg/ml and resuspended in histamine 5×10^{-4} M.
a. Release of histamine on suspension of granules in distilled water.
b. Release of histamine on suspension in heparin 500 µg/ml.
■ Histamine rest before incubation in histamine.
□ Total histamine uptake after incubation in histamine 5×10^{-4} M.
▨ Histamine release on washing incubated granules in dist. water.

pH was adjusted by mixtures of NaCl, HCl and sodium phosphate buffer, care being taken to keep the sodium concentration constant at 10^{-3} M. Fig. 9 shows the pH dependence of the histamine uptake which seemed to reach a maximal level around pH 7. The histamine taken up was again totally released on suspension of the granules in isotonic sodium chloride solution.

Fig. 10 shows the release curve of histamine refilled granules upon exposure to various sodium concentrations. Granules isolated with Ederol No. 14 filter paper

were incubated in a mixture of histamine dihydrochloride and histamine base to give a pH of 6 5 and a total histamine concentration of 5×10^{-4} M at 20° for 15 min After washing in distilled water the granules were divided into 5 samples and exposed to sodium chloride All histamine was released at 10^{-1} M NaCl

The experiment summarized in Fig 11 was performed in order to determine the influence of time and temperature on the re-uptake of histamine into granules "Free granule" sediments from water-lysed mast cells were exposed to heparin (500 μg/ml), then washed with water and, after recentrifugation, incubated in a mixture of histamine base and histamine dihydrochloride to give a pH 7 and a total histamine concentration of 5×10^{-4} M After washing the sediments in distilled water at 0° C, the histamine uptake was determined at various temperatures No significant differences were observed within the temperature range 0—37° C, the uptake being practically maximal even after incubation for 1 min at 0° C

The refilled histamine stores retained about 80 per cent of their histamine on resuspension in distilled water On exposure to isotonic sodium chloride all the histamine was immediately released, as was shown to be the case with the original granular histamine (See Fig 3)

Discussion

Two main hypotheses have been proposed to explain the mode of storage of mast cell histamine 1) Histamine is enclosed in 'a free diffusible form in a particle with a lipid boundary Release of histamine is dependent upon osmotic changes or lytic factors, which cause a rupture of the granular membrane or an increase in its permeability 2) Histamine is electrostatically linked to heparin Release of histamine requires an exchange, at granular sites between histamine and cations

It is true that a minimum of 20 per cent of the cellular histamine occurred "free" in the suspension fluid after the water lysis of mast cells but as will be discussed below, this "free' histamine might be explained as an experimental artifact

The present observations agree with the idea that mast cell histamine is electrostatically bound to some granular matter The granules retain histamine in spite of repeated suspension in distilled water A lipid membrane would have been serious ly damaged by such procedures allowing a total escape of the histamine if it were stored in "free diffusible form On the other hand histamine is immediately released in the presence of adequate extragranular concentrations of cations or a complex binding anion (heparin Granular material is able to take up histamine especially when the histamine stores are depleted This new granular histamine is probably retained by the same mechanism as the original histamine As is the case with the non-depleted granular stores, the refilled stores withstand washing in distilled water but are immediately depleted on the addition of cations *e g* isotonic sodium chloride solution

It could be argued that granules isolated in distilled water do not represent a normal granular material since they swell in distilled water and shrink with a increasing tonicity of the suspension fluid unpublished observations) How

they retain, release, and take up histamine in an identical manner irrespective of their suspension in water or isotonic sucrose, indicating that swelling due to suspension in water does not cause any *profound changes in the granular storage mechanism*.

The release and the uptake of histamine in the granular stores showed a dependence on the extragranular concentrations of inorganic cations, heparin and histamine, as would be expected with a passive exchange between intra- and *extragranular* ions. Accordingly, both processes occur rapidly even at 0° C.

All reports based on electron microscopic studies agree that the mast cell granules *intracellularly* are enclosed between membranes *belonging to the intracellular reticulum*. However, *the granules seem to lack a definitive membrane, since no membrane* can be observed around *extracellularly* located granules. The rapid passage of histamine and cations in and out of such granules agrees well with the absence of a lipid boundary. Accordingly, to explain the histamine holding capacity of the *intracellular* granules on an electrostatic basis, one has to assume that *intracellularly* the reticular membranes embracing the granules *form an effective barrier against* cellular water and ions.

In the absence of exact knowledge of the chemical structure of the granular material, and until adequate quantitative data are available, the nature of the histamine binding sites can only be a matter of speculation. The granules are reported to contain protein and heparin but no significant lipid or nucleic acid phosphorous. The molar ratio of histamine adenosine triphosphate (ATP) was found to be 200—250/1 (Johansson and Uvnäs, unpublished), a fact which excludes ATP as the histamine binding site. Heparin is a clear candidate for the acid moiety that binds histamine. Several *in vitro* studies have demonstrated its ability to combine reversibly with histamine. Calculated on a weight basis, a heparin/histamine ratio around 4 : 1 suffices to form a stable water soluble complex. Histamine is released from such a complex in the presence of cations, e.g. sodium ions.

From determinations of the sulphate content of our mast cells we have estimated the heparin content as about 70 μg/10^6 cells (Bergendorff and Uvnäs, unpublished), a figure in fair agreement with the result of other investigators. The histamine content has been determined as 10—15 μg (base) per 10^6 cells. To judge from the *in vitro* observations mentioned above, the calculated intracellular heparin/histamine ratio of 5—6 : 1 should suffice for an effective binding of histamine in the granules. However, the capacity of heparin to complex *with histamine in vitro is dependent* on the sulphur containing acid groups of the heparin molecule. Desulphated heparin is unable to form a stable complex with the amine (Kobayashi 1962). It is doubtful whether the SO_4 groups of the heparin are free *to bind histamine intracellularly*. The nature of the heparin protein linkage is not established, but observations indicate the linkage to be electrostatic. In that case a binding of the SO_4 groups of heparin to amino groups of the protein may be a reasonable assumption. If this is so the only alternative binding sites for the histamine are the COO groups of the heparin or the protein. The weak electrostatic binding of the histamine and the pH dependence of this binding, as demonstrated in the present experiments, argue in

favour of a binding of histamine to weak acid groups Further experiments are needed to localize such groups within the heparin protein complex

On lysis of the mast cells a minimum of 20—25 per cent of the histamine occurred "free in the suspension fluid Does this histamine represent a free" histamine fraction in the mast cell or is it an experimentally produced artifact? In our opinion the "free' histamine fraction is an experimental product for the following reasons

On lysis of the mast cells the granules are suddenly stripped of their protective intracellular membranes and exposed to the physical strain of being suspended in a great volume of water The fact that only 20—25 per cent of the granular histamine is released illustrates the strong affinity of the histamine for the granular matter There is a more than thousandfold concentration gradient existing between the intra and extragranular histamine (the granules supposed to be spheres with a diameter of 0 1 μ) The occurrence of "free histamine from lysed mast cells is no doubt partly due to the dissolution of granular matrix (5—10 per cent) and partly due to the outflow of intracellular cations The rest of the free histamine might well be explained as due to the dissociation in the water of the ionic binding between histamine and the heparin protein complex

The granules from rat peritoneal mast cells offer a particularly favourable system for the study of the histamine storage mechanism These granules are almost insoluble in water, and *in vivo* they can be seen dispersed in the tissues for hours after the degranulation of mast cells In contrast in other species such as the guinea pig the granules dissolve rapidly after discharge from the mast cells *in vivo* In such animal species with mast cells containing water soluble granular matter it will be more difficult to establish experimentally the mode of intragranular binding of histamine However, a water soluble protein heparin complex should be able to store histamine, provided that the complex is effectively shielded intracellularly from cellular water and ions *e g* by intracellular reticular membranes

Several previous investigators have isolated histamine carrying metachromatic particles from homogenized tissues dog liver guinea pig lung) mast cell tumours (mouse) and peritoneal mast cells rat Such particles have been observed to lose much (sometimes most of their histamine during the isolation procedures On the other hand such isolated particles have also been reported to stubbornly retain histamine even on suspension in cation containing solutions such as isotonic phosphate buffer Tyrode s solution etc These observations seemingly contradict the present findings on the storage properties of our isolated mast cell granules The explanation might be that in the previous experiments cited the histamine carrying particles were isolated by homogenization and differential centrifugation under experimental conditions that often involved hours of suspension in isotonic or hypertonic sucrose solutions As mentioned under Methods we have observed that prolonged exposure of the granular matter from mast cells to sucrose may change the storing qualities of the granules as observed in the above cited experiments High losses of histamine occur during isolation and a cation resistant histamine rest appears in such sucrose exposed granules

Conclusions

The present results agree with the assumption that in rat peritoneal mast cells the histamine is electrostatically bound to a granular protein-heparin complex The granules retain their histamine on suspension in water but immediately dispose of it in the presence of cations Granules deprived of their histamine take up the amine anew when suspended in histamine-containing cation-poor solutions

The rapid passage of histamine and cations into and out of the granular sites and also the apparent temperature independence of these processes are in accordance with the absence of a definitive granular membrane It has to be assumed that intracellularly the ability of the granules to take up and retain histamine is dependent on the presence of reticular membranes that protect the granules and their *binding sites from cellular water and ions Outside the cells, on the other hand, the* granules will immediately lose all their histamine to the surrounding fluid due to the ionic strength of the extracellular medium

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Release of ^{3}H-Metaraminol by Different Mechanisms[1]

By

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Abstract

CARLSSON, A and B WALDECK *Release of ^{3}H-metaraminol by different mechanisms.* Acta physiol scand 1966 *67* 471—480

Mice were given ^{3}H metaraminol i v After 15 min, when the uptake by the adrenergic nerve fibres had been almost completed, various drugs were given and their ability to release the amine was investigated Protriptyline and chlorprothixene, which block the amine-concentrating mechanism at the level of the cell membrane caused a clearcut release of ^{3}H-metaraminol This was true also of

α Methyl metatyrosine has been found to undergo decarboxylation and subsequent β-hydroxylation *in vivo* to form metaraminol, which then causes displacement of noradrenaline from tissue stores (Carlsson and Lindqvist 1962) After administration of metaraminol this amine, like noradrenaline, accumulates in tissues and this accumulation has been shown to occur in the adrenergic nerves (Anden 1964, Shore, Busfield and Alpers 1964, Almgren 1965, Almgren and Waldeck 1966) The accumulation of metaraminol can be inhibited by a variety of drugs, *e g* imipramine and derivatives, cocaine and guanethidine (Shore *et al* 1964, Carlsson and Waldeck 1965a), drugs well known to inhibit accumulation of ^{3}H noradrenaline in the tissues (Axelrod, Hertting and Potter 1962) Metaraminol accumulated in adrenergic nerves is retained over a period of many days (Anden 1964) It can be released by sympathetic nerve stimulation (Crout *et al* 1964), and decentralisation of the nerves results in a slower disappearance of the amine (Almgren 1965, Almgren and Waldeck 1966) Drugs known to release noradrenaline, *e g* reserpine, guanethidine and

[1] This investigation has been presented in part at the "International symposium on Mechanisms of release of biogenic amines" (Carlsson 1966) and in a preliminary communication (Carlsson and Waldeck 1965 b)

tyramine, have also been found to release metaraminol (Shore *et al* 1964, Carlsson and Waldeck 1965b)

As first postulated by Hillarp and coworkers (Carlsson, Hillarp and Waldeck 1963) the ability of adrenergic nerves to concentrate amines appears to depend on at least two separate mechanisms, one located at the level of the cell membrane, the other in the specific amine-storing granules The first-mentioned mechanism is selectively blocked by cocaine, imipramine (and its congeners, *e g* desipramine and protriptyline), and chlorprothixene, and the second by *e g* reserpine and prenyl amine (Hillarp and Malmfors 1964, Dahlstrom, Fuxe and Hillarp 1965, Malmfors 1965, *Carlsson and Waldeck 1965 a, b and unpublished observations*)

In the present investigation ^{3}H metaraminol is used to elucidate different mechanisms of drug induced amine release Metaraminol which has the advantage of being resistant against monoamine oxidase (MAO) as well as catechol 0 methyl transferase, appears to be a valuable tool in the investigation of adrenergic mechanisms It must be kept in mind, however, that although metaraminol seems to utilize the same transport and storage mechanisms as noradrenaline, certain differences in *e g affinity may exist*

Material and methods

Mice divided at random into groups of 6 were used and all experiments were carried out at 30 C The animals received ^{3}*H metaraminol* 0 02 mg/kg i v preceded and or followed by the drugs to be tested After sacrifice the hearts (and in some cases the femoral muscles were removed pooled and homogenized in 0 4 N perchloric acid The extracts were chromatographed on DOWEX 0W X4 ion-exchange resin and the radioactivity determined by liquid scintillation counting The analytical procedure has been described in detail earlier (Carlsson and Waldeck 1963 1965 c

The following drugs were used ( ) Amphetamine sulphate commercial) (+)-amphetamine [illegible] guanethidine phosphate *Hoffmann La Roche bufotenine monooxalate [illegible]

[illegible] creatinine sulphate Sigma Chemical Company [illegible]

chloride (*Dr [illegible] Ltd), (±) normetanephrine hydrochloride (*Winthrop Products) pargyline (*Dr G M Everett Abbott Laboratories) prenylamine lactate (Segontin® *Dr H Heisse Hoechst Anil n Ltd protriptyline hydrochloride *Dr C A Stone Merck Institute for Therapeutic Research reserpine (*Serpasil*® *The Swedish Ciba Ltd) tranylcypromine sulphate (*Smith Kline & French [illegible] hydrochloride (Sigma Chemical Company and p-tyramine hydrochloride Bio- [illegible] as prepared [illegible] nd Waldeck [illegible] e gratefully

Results

1 *Release of ^{3}H metaraminol by protriptyline*

Protriptyline, 10 mg/kg, was given to mice i v 15 min after the i v administration of ^{3}H metaraminol and then 5 mg/kg every *2 hrs Controls* were given ^{3}H metaraminol alone The animals were killed at various time intervals and ^{3}H metaraminol

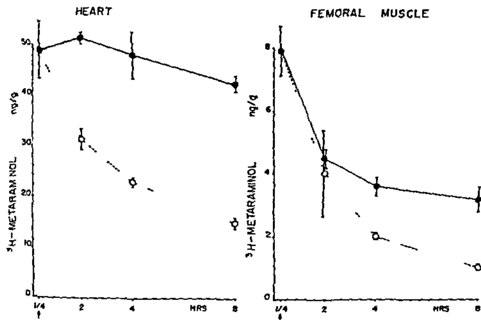


Fig 1 Rel ... ent

in the heart and femoral muscle was assayed After protriptyline the ³H metaraminol level in the heart fell more rapidly than in the controls and after 8 hrs only about 30 per cent of the corresponding control value was left (Fig 1) In the femoral muscle protriptyline caused a similar decrease although ³H metaraminol in the controls disappeared more rapidly during the first 2 hrs than it did in the heart Also here the ³H metaraminol level after 8 hrs was about 30 per cent of the correspond ing control value The difference between heart and femoral muscle is probably due to the fact that the latter tissue has a relatively poor supply of adrenergic nerves Consequently extraneuronal uptake becomes a relatively important factor initially (Carlsson and Waldeck 1965 c

2 *Release of ³H metaraminol by reserpine*

Reserpine 0 5 mg kg i v) was given 15 min after the administration of ³H metarami nol and the animals were killed after another 1 3 4 or 3 3 4 hrs Controls received ³H metaraminol only After reserpine ³H metaraminol in the heart started to disappear and after 1 3/4 hrs there remained about half and after another 2 hrs about one third of the correspond ng control value Fig 2 Chlorisondamine a ganglionic blocking agent given i v in a dose of 10 mg kg 15 min after the administration of ³H metaraminol had no significant influence on the disappearance of ³H met

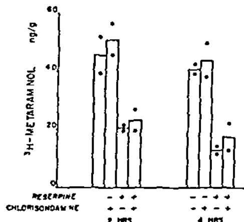


Fig 2 Release of ³H metaraminol from heart of mice by reserpine and the effect of chlorisondamine
Reserpine 0 5 mg kg and or chlorisondamine 10 mg kg were injected i v 15 min after 0 02 mg/kg ³H metaraminol The animals were killed 2 and 4 hrs after ³H metaraminol Each point represents one experimental group consisting of 6 mice

minol, nor did it appreciably effect the rate of reserpine induced release of ³H metaraminol (Fig 2)

3 Release of ³H metaraminol after combined treatment with drugs with different sites of action

Mice were injected i v with ³H metaraminol, 15 min later followed by the drugs to be tested These were protriptyline 10 or 20 mg/kg, chlorprothixene 10 mg kg, *reserpine 0 5 or 1 mg/kg prenylamine 10 mg/kg and guanethidine 10 mg kg All* injections were made i v The animals were killed 15 or 45 min after the administration of the drugs Given alone protriptyline chlorprothixene reserpine and prenylamine caused a moderate reduction of ³H metaraminol in the heart (Fig 3a compare also Fig 1 and 2) It should be noted that doubling the dose of protriptyline or

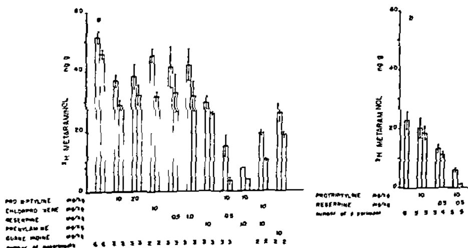


Fig 3 Release of ³H metaraminol from heart of mice by drugs with different sites of action The drugs were given i v a) 15 min and b) 3 days after 0 02 mg/kg ³H metaraminol The left column of each pair represents the amine level 15 min and the right 45 min after the administration of drugs The data are the means ± SEM Each experimental group consists of 6 mice

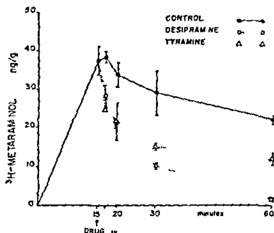


Fig 4 Release by desipramine and tyramine of ³H metaraminol accumulated in the heart of ...

desipramine 10 mg/kg or tyramine 10 mg/kg were injected i v (indicated by the arrow) Controls received ³H metaraminol only The data are the means of 3—5 experimental groups ± SEM Each experimental group consists of 6 mice

reserpine did not result in an increased rate of release The reduction induced by guanethidine seemed to be slightly more pronounced than that produced by the other drugs, at least 45 min after the administration In combination, however, protriptyline and reserpine, or protriptyline and prenylamine, or chlorprothixene and prenylamine caused a rapid and pronounced reduction of ³H metaraminol

In a similar experiment mice received ³H metaraminol i v 3 days before the i v injection of protriptyline, 10 mg/kg, or reserpine, 0 5 mg/kg, or a combination of both Controls received ³H metaraminol alone The animals were killed 15 or 45 min after the drug administration In contrast to the experiment where ³H metaraminol was given shortly before the drugs to be tested, the ³H metaraminol reduction caused by protriptyline alone was not significant (Fig 3b) Reserpine, however, in 45 min reduced the ³H metaraminol level in the heart to half of the control value, and the two drugs in combination caused the amine to disappear almost completely

4 *Release of ³H-metaraminol accumulated in the heart of animals pretreated with reserpine* Reserpine, 10mg/kg, was given i p to mice 6 hrs before the administration of ³H metaraminol Fifteen minutes later desipramine 10 mg/kg or tyramine, 10 mg/kg, were given i v Controls received reserpine and ³H metaraminol alone The animals were killed at various time intervals and the ³H metaraminol level in the heart was estimated Fifteen minutes after the injection of ³H-metaraminol 37 8 ng/g of this amine were found (Fig 4) From 15 to 60 min the amine level fell some 40 per cent in the controls Desipramine and tyramine considerably accelerated the disappearance of ³H metaraminol Thus within 15 min the amine level dropped to less than half the corresponding control value During the first 5 to 15 min after the injection of desipramine or tyramine there seemed to be little or no difference in activity between the two drugs However, the effect of tyramine appeared to be short lasting, probably owing to its rapid elimination by MAO, whereas the effect of ... was of longer duration

TABLE I. Release by various drugs of ^{3}H metaraminol accumulated in the heart of mice pretreated with reserpine.

Mice pretreated with reserpine (10 mg/kg i.p. 6 hrs before) were given 0.02 mg/kg ^{3}H metaraminol i.v. Fifteen minutes later the drugs to be tested were given i.v. and the animals sacrificed after another 15 min. Controls receiving reserpine and ^{3}H metaraminol were run in parallel. The average of 12 control groups was 32.8 ± 8.4 ng/g (SD). The data are calculated as *per cent of this value*. Each experimental group consists of 6 mice.

Dose mg/kg	Drug	Average	Range	Number of experimental groups
10	Azapetine	82		1
	Bufotenine	75		1
	N-Ethylnormetaoxedrine	58	54–62	2
	Guanethidine	49	39–55	4
	Hordenine	67	65–68	2
	5-Hydroxytryptamine	80		1
	Isoprenaline	94	90–98	2
	3-Methoxytyramine	73	68–79	2
	Normetanephrine	80		1
	Prenylamine	69	51–86	4
	Tryptamine	61	54–67	2
5	(±) Amphetamine	30	25–34	2
	(+) Amphetamine	28	23–33	2
2	(−) Metaraminol	[1]28	21–34	4
	(+) Metaraminol	[2]43	40–48	4
	Reserpine	126	113–139	2

[1] ±3 (SEM)
[2] ±2 (SEM)

Mice pretreated with reserpine as above received various drugs i.v. 15 min after ^{3}H-metaraminol. A group treated with reserpine and ^{3}H-metaraminol served as control. After another 15 min the animals were killed and ^{3}H-metaraminol in the heart determined. Since the material is limited, only relatively pronounced effects can be regarded as certain. (−) Metaraminol, (+)-amphetamine, and (±) amphetamine, were found to be most efficient in releasing ^{3}H-metaraminol (Table I). (+)-Metaraminol was less efficient than (−)-metaraminol ($P < 0.01$). Among the other drugs tested only guanethidine appeared to be able to release as much as 50 per cent of ^{3}H-metaraminol within 15 min.

5. *Release of ^{3}H-metaraminol by monoamine oxidase inhibitors*

Mice were given the MAO inhibitor nialamide, 100 mg/kg i.p., 2 hrs before ^{3}H-metaraminol. At various time intervals the animals were killed and ^{3}H-metaraminol in the heart assayed. Fifteen minutes after the administration of ^{3}H metaraminol the level of this amine in the heart was about 80 per cent of that of a corresponding

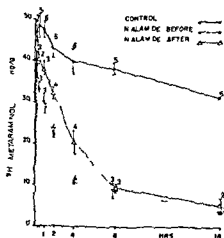


Fig 5

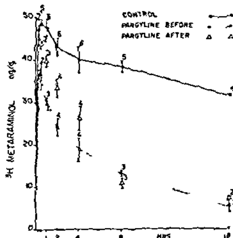


Fig 6

Fig 5 Effect of nialamide on the disappearance of ³H metaraminol from the mouse heart Nialamide 100 mg/kg was given i p 2 hrs before or 15 min after the i v injection of 0 02 mg kg ³H metaraminol Controls received ³H metaraminol only The data are the means ± SEM The number of experimental groups is presented at each point Each experimental group consists of 6 mice

Fig 6 Effe ...

TABLE II Effect of nialamide on the accumulation of ³H metaraminol in the mouse heart.

Nialamide 100 mg kg was given i p various times before the i v administration of 0 02 mg kg ³H metaraminol and the animals were killed 30 min after this time The controls received ³H metaraminol alone Each experimental group consists of 6 mice

	³H metaraminol ng g
Control	48 7 3 0
Nialamide 2 hrs before	33 2 1
Nialamide 6 hrs before	41 9
	40 9
Nialamide 18 hrs before	40 ›
	31 4

¹ Mean SEM (from fig 5

control group not receiving nialamide Fig 5 Then the ³H metaraminol continued to disappear and 18 hrs after the administration the concentration was only about 10 per cent of the corresponding control value When nialamide was given 15 min after ³H metaraminol a similar curve was obtained though with a delay of about 2 hrs On the other hand when nialamide had been given 6 or 18 hrs before ³H-metaraminol the amine uptake was about the same as when nialamide had

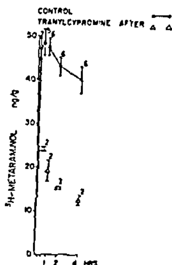


Fig. 7. Effect of tranylcypromine on the disappearance of ³H-metaraminol from the mouse heart. Tranylcypromine 10 mg/kg was given i.v. 15 min after the i.v. injection of 0.02 mg/kg ³H-metaraminol. The control material is the same as in Fig. 5. The data are the means ± S.E.M. The number of experimental groups is presented at each point. Each experimental group consists of 6 mice.

given 2 hrs before (Table II). Similar experiments where nialamide was replaced by the MAO inhibitor pargyline, 100 mg/kg i.p., gave essentially the same results (Fig. 6).

A third MAO inhibitor, tranylcypromine, was also tested for its ability to release ³H-metaraminol. Tranylcypromine, 10 mg/kg, was given i.v. 15 min after ³H-metaraminol. In 15 min tranylcypromine caused a drop of ³H-metaraminol in the heart to 50 per cent of the control value and after another 3 1/2 hrs only about 25 per cent was left as compared to the corresponding control value (Fig. 7). This tranylcypromine-induced release was more rapid than that caused by the other MAO inhibitors, which may be related to its amphetamine-like structure and activity.

Also the MAO inhibitors iproniazid, MO 1255, and harmaline were found to release ³H-metaraminol under the conditions described above (data not shown).

Discussion

The present investigation has shown that ³H-metaraminol taken up by adrenergic nerve fibers can be released by drugs known to block one of the two amine-concentrating mechanisms of these fibers. One of these mechanisms appears to be located at the level of the cell membrane and is selectively blocked *i.a.* by agents *belonging to the imipramine group, e.g.* protriptyline and desipramine. The other mechanism is located in the specific amine-storing granules and is selectively blocked *e.g.* by reserpine and prenylamine. Furthermore, it has been shown that the combination of the two types of agents causes a much more rapid release than either type alone, even in maximum dosage. This was true irrespectively of whether the ³H-metaraminol had been given 15 min or 3 days before the releasing agents, indicating that irreversible fixation of the amine does not occur in the adrenergic nerve fibres. However, this does not exclude the possibility of a slow movement

of the amine from labile to more stable pools In favour of such an assum we found that protriptyline was less efficient in releasing the amine at the compared to the earlier interval Further, Crout *et al* (1964) have observed electrical stimulation at high frequencies of adrenergic nerves results in a m r pronounced release of metaraminol at short (2 hrs) compared to longer (17—2(hrs) intervals after the administration of this amine If these observations are to be interpreted in terms of movement into a more stable pool it must be concluded from our observations with reserpine that this agent is capable of releasing the amine from both pools equally well It should be recalled that different pools of catecholamines have been observed in the amine storing granules of the adrenal medulla (Hillarp 1960)

Guanethidine caused a more rapid release than either reserpine or protriptyline given alone This probably means that guanethidine blocks both the above mentioned amine concentrating mechanisms Evidence in favour of this interpretation has been presented earlier (Carlsson and Waldeck 1965c)

When ³H metaraminol is given to animals pretreated with reserpine, the amine is still taken up by the nerve fibers but is not retained very efficiently The uptake is probably caused by the amine concentrating mechanism at the level of the cell membrane which is not sensitive to reserpine The poor retention is probably due to blockade of the amine storing mechanism of the granules (see Carlsson and Waldeck 1965c) In the present investigation we studied the ability of various agents to release the amine thus concentrated in the cytoplasm outside the granules Blockade of the membrane mechanism by desipramine resulted in rapid release In addition a number of analogues of the adrenergic transmitter proved active, namely tyramine metaraminol and amphetamine It might be tempting to suggest that the activity of these analogues in this respect is due to blockade of the membrane mechanism Although the present data do not exclude this possibility recent observations point in another direction Amphetamine in the dose employed in the present experiments causes but a moderate blockade of the membrane mechanism as judged by previous uptake experiments in animals not pretreated with reserpine (Carlsson and Waldeck 1965a) Further these analogues differ from drugs like protriptyline and desipramine in ability to release extragranular noradrenaline The former compounds are much more active Malmfors 1965 Carlsson and Waldeck 1966) These observations suggests that the analogues in question possess releasing activity unrelated to blockade of the amine concentrating mechanism of the cell membrane Such activity might involve displacement of noradrenaline from hypothetical binding sites within the nerve fibre but presumably outside the granules (cf Furchgott *et al* 1963 or alternatively a change in the cell membrane leading to increased release

While studying the effect of tyramine whose activity we wished to prolong by MAO inhibition we accidentally observed that the MAO inhibitors themselves are capable of releasing metaraminol from the adrenergic nerve fibres The that this effect does not show up immediately suggests that it is indirect It is t

ing to suggest that the effect is mediated by monoamines accumulating in the adrenergic nerves as a consequence of MAO inhibition Unpublished experiments show that the releasing activity of MAO inhibitors is blocked by decarboxylase inhibitors, which presumably block the formation of monoamines sufficiently to prevent their accumulation

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Biochemical and Histochemical Studies on the Effects of Imipramine-like Drugs and (+)-Amphetamine on Central and Peripheral Catecholamine Neurons

By

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Abstract

Carlsson, A., K. Fuxe, B. Hamberger and M. Lindqvist *Biochemical and histochemical studies on the effects of imipramine like drugs and (+)-amphetamine on central and peripheral catecholamine neurons* Acta physiol scand 1966 67 481—497

Rats were depleted of tissue monoamines by reserpine treatment and were then treated with a monoamine oxidase inhibitor (nialamide) Subsequent injection of L-dopa caused accumulation of [illegible]

No effect on DA fibres was observed *In vitro* studies on tissue slices showed a blocking action of [illegible]

fibres

Besides the monoamine-oxidase (MAO) inhibitors two important groups of central stimulants have been shown to interact with catecholamine (CA) neurons, namely the antidepressive drugs of imipramine type and the amphetamine group The former group, particularly congeners with only one methyl group on the [illegible] e g desipramine and protriptyline, do not influence normal tissue [illegible]

amines but potentiate and prolong various actions of noradrenaline (NA) and adrenaline as well as the effects of sympathetic nerve stimulation (Sigg 1959, Haefely, Hürlimann and Thoenen 1964, Stone *et al* 1964) These effects are brought about by inhibition of an important mechanism for inactivation of CA, *viz* uptake by adrenergic nerve fibres (Hertting, Axelrod, Kopin and Whitby 1961, Hertting, Axelrod and Whitby 1961, Axelrod, Hertting and Potter 1962, Axelrod 1965) This uptake mechanism, which is resistant to reserpine, has been shown to be located at the level of the nerve-cell membrane and should be distinguished from the reserpine-sensitive amine-concentrating mechanism of the intracellular storage granules (Carlsson, Hillarp and Waldeck 1963, Hamberger *et al* 1964, Hillarp and Malmfors 1964, Muscholl 1965, Malmfors 1965, Carlsson and Waldeck 1965 a, b, c) *In-vitro* experiments have shown that imipramine-like drugs block the uptake of ^{3}H-NA by slices of brain and other tissues (Titus and Spiegel 1962, Dengler 1965) Subsequent experiments, using the histochemical fluorescence method of Hillarp and coworkers, have directly demonstrated uptake of NA and α-methyl-NA by central and peripheral CA nerve fibres *in vitro*, as well as the blocking action of *i a* imipramine-like drugs (Hamberger and Masuoka 1965, Hamberger 1966 to be published) *In-vivo* experiments have shown that terminals and non-terminal axons of central dopamine (DA) neurons located outside the blood-brain barrier (in the median eminence) are able to take up and concentrate circulating CA (Fuxe and Hillarp 1964) Otherwise his barrier has hampered the investigation of central CA neurons *in vivo* with respect uptake mechanisms However, after specific blood-brain barrier lesions, a reserpine-resistant uptake of CA has been demonstrated in CA nerve terminals (Hamberger and Hamberger 1966)

Like the imipramine agents, amphetamine and related drugs do not influence normal tissue CA levels except in high dosage They differ in pharmacological actions from the imipramine group in several important respects They have little antidepressant activity but rather act as psychomotor stimulants Peripherally they are generally classified as indirectly acting sympathomimetics, *i e* they depend on an intact sympathetic nervous system for their activity Reserpine has been reported to prevent their peripheral actions (Burn and Rand 1958) The central actions are evidently not blocked by reserpine This has lead to speculations that amphetamine acts directly on receptors, particularly 5 hydroxytryptamine (5 HT) receptors (Vane 1960, Innes 1963), but this is unlikely because inhibitors of CA synthesis have been shown to block the actions of amphetamine (Weissman and Koe 1965, Hanson 1966, Randrup and Munkvad 1966)

In the present investigation a combined biochemical and histochemical approach is utilized to further elucidate the effects of the two groups of drugs on peripheral and particularly central CA neurons Advantage is taken of the fact that the CA precursor L-dopa, unlike the CA themselves, is able to penetrate through the blood brain barrier and thus to cause accumulation of CA in the brain (Carlsson, Lindqvist and Magnusson 1957, Carlsson *et al* 1958) The *in-vivo* data thus obtained are related to observations on tissue slices *in vitro* Part of this invesigation has been

TABLE I Drug treatments of animals used for histochemical studies

All animals were pretreated with reserpine (10—15 mg/kg i p) and nialamide (100 mg kg i p) 20—24 and 4—6 hrs before sacrifice respectively The test drugs were given i p 45 min and L-dopa s c 30 min before sacrifice

Number of animals	Desipramine mg/kg	Protriptyline mg kg	Lu 3 010 mg/kg	(+) Amphetamine mg/kg	L-dopa mg kg
10					10
8					25
[illegible]					50
4	25				10
4	25				25
4	25				50
4		25			25
4		25			50
4			25		50
4			25		25
4			10		25
4				5	10
6				7 5	[illegible]
4				5	50
3				3 5	25
5				0 5	25
4				0 15	25

reported in preliminary publications (Carlsson Dahlstrom Fuxe and Lindqvist 1965 Carlsson, Lindqvist, Dahlstrom Fuxe and Masuoka 1965 Carlsson *et al* 1966

Material and Methods

Biochemistry The experiments were performed on adult male Sprague-Dawley or Wistar rats (200—300 g) The treatments of the animals are described in Table II—VII

The animals were killed by decapitation and amine analyses were performed on brain and heart Noradrenaline was determined by the method of Bertler Carlsson and Rosengren 1958 and dopamine by the method of Carlsson and Waldeck 1958 modified by Carlsson and Lindqvist (1962 a Normetanephrine (NM) was estimated by the method of Carlsson and Lindqvist (1962 b) and 3-methoxytyramine (MT) by the method of Carlsson and Lindqvist 1962 b 1963 cf Carlsson and Waldeck 1964

Histochemistry Male albino Sprague Dawley rats 200 300 g were used

In vivo experiments Rats were treated with drugs according to Table I This type of experiment was performed to test the ability of the various drugs desipramine protriptyline Lu 3-010 (+) amphetamine) to prevent the dopa induced accumulation of amines in central DA and NA neurons of rats pretreated with reserpine and nialamide

A second type of experiment was set out to study the effect of (+ amphetamine (5 mg kg i p 1 hr before sacrifice on the increased intraneuronal amine levels observed in rats pretreated with nialamide (700 mg/kg i p 4 hrs before sacrifice) Ten experimental and 5 control animals receiving nialamide alone were examined

The histochemical fluorescence method for the demonstration of DA NA and 5 HT at the cellular level was used (Falck *et al* 1962 Falck 1962 see review by Hillarp Fuxe and Dahlstrom 1966 The rats were killed by decapitation under light chloroform anesthesia The medulla oblongata pons, mesencephalon diencephalon and the caudal half of the telencephalon and parts of the thoracic spinal cord were dissected out freeze-dried treated with formaldehyde gas and [illegible] ted in

TABLE II Effect of various doses of desipramine, protriptyline and Lu 3 010 on L-dopa induced noradrenaline accumulation in rat brain and heart

The test drugs (desipramine, protriptyline or Lu 3 010) were given 45 min and L-dopa 30 min before sacrifice The rats were pretreated with reserpine (10 mg/kg i p) and nialamide 22 and 4 hrs before sacrifice respectively Control rats were treated in the same way as the experimental animals except that no test drug was given

The values are means ± standard errors of the means, expressed in μg/g tissue Figures in brackets indicate number of experiments Each experiment was performed on 2 or 3 pooled organs

Drugs mg/kg			Desipramine		Protriptyline		Lu 3 010	
Nialamide i p	Test drug i p	L-dopa i v	Heart	Brain	Heart	Brain	Heart	Brain
100	25	25	0 04 (4) ±0 006	0 05 (4) ±0 009	0 03 (1)	0 06 (1)	0 04 (2) ±0 005	0 13 (2) ±0 020
100	10	25	0 05 (4) ±0 003	0 10 (4) ±0 005	0 04 (2) ±0 005	0 07 (2) ±0 010	0 04 (4) ±0 005	0 14 (4) ±0 016
100	5	25	0 05 (3) ±0 006	0 14 (3) ±0 024	0 05 (2) ±0 005	0 10 (3) ±0 015	0 07 (2) ±0 005	0 16 (2) ±0 015
100	2 5	25	0 06 (2) ±0 010	0 16 (2) ±0 005	0 07 (2) ±0 005	0 16 (2) ±0 005	0 09 (2) ±0 025	0 24 (2) ±0 035
100	1	25	0 08 (2) ±0 005	0 19 (2) ±0 035	—	—	—	—
100	0	25	0 10 (31) ±0 004	0 15 (22) ±0 007	0 10 (31) ±0 004	0 15 (22) ±0 007	0 10 (31) ±0 004	0 15 (22) ±0 007
50	25	50			—	0 05 (2) ±0 005		—
50	10	50			0 06 (1)	0 06 (1)	0 04 (1)	0 15 (1)
50	0	50			0 10 (4) ±0 010	0 17 (10) ±0 015	0 10 (4) ±0 010	0 17 (10) ±0 015

paraffin sectioned and mounted as previously described in detail (Dahlstrom and Fuxe 1964 Hamberger Malmfors and Sachs 1965) The heart auricles the vas deferens and the submaxillary gland were also studied

from cortex, caudate nucleus-putamen hypothalamus tegmentum and vas deferens The slices were then moved to fresh medium and preincubated in a shaker at 37° C for 15 min in the presence of the drugs to be investigated (desipramine 300—0 003 μg ml protriptyline 30—0 03 μg/ml

—0 02 μg/ml) Then α methyl NA 1—0 03 μg/ml

min In two experiments the animals were injected

Feed and

rate experiments were performed identically

TABLE III Effect of desipramine (25 mg/kg i p) on amine accumulation in rat brain and heart following various doses of L-dopa

Desipramine was given 45 min and L-dopa 30 min before sacrifice The rats were pretreated with reserpine (10 mg/kg i p) and nialamide 22 and 4 hrs before sacrifice, respectively Control rats were treated in the same way as the experimental animals except that no desipramine was given

The values are means ± standard errors of the means, expressed in μg/g tissue Figures in brackets indicate number of experiments Each experiment was performed on 2 or 3 pooled organs

Drugs mg/kg			Heart		Brain		
Nialamide i p	Desipramine i p	L-dopa s c	Noradrenaline	Dopamine	Noradrenaline	Dopamine	3-Methoxytyramine
50	25	50	–	–	0 05 (4) ±0 023	1 86 (4) ±0 253	1 56 (4) ±0 169
50	0	50	0 10 (4) ±0 010	5 71 (3) ±1 502	0 17 (10) ±0 015	1 54 (10) ±0 134	1 20 (9) ±0 092
100	25	50	0 10 (2) ±0 005	5 57 (2) ±0 695	0 12 (2) ±0 040	2 21 (2) ±0 195	1 68 (2) ±0 660
100	0	50	0 12 (2) ±0 005	4.20 (2) ±0 140	0.20 (2) ±0 000	2 04 (2) ±0 015	1 80 (2) ±0 190
100	25	25	0 04 (4) ±0 006	1 95 (2) ±0 620	0 05 (4) ±0 009	0 90 (4) ±0 248	1 19 (2) ±0 305
100	0	25	0 10 (31) ±0 004	1 87 (24) ±0 109	0 15 (22) ±0 007	1 24 (21) ±0 048	0 92 (13) ±0 060
100	25	12 5	0 03 (4) ±0 003	0 79 (3) ±0 093	0 03 (3) ±0 009	0 37 (3) ±0 115	0 42 (3 ±0 132
100	0	12 5	0 08 (3) ±0 012	0 76 (3) ±0 077	0 13 (3) ±0 029	0 59 (3 ±0 104	0 36 (3) ±0 111
200	25	12 5	0 03 (1)	0 42 (1)	0 05 (1)	0 47 (1)	0 51 (1
200	0	12 5	0 06 (1)	0 53 1	0 17 (1)	0 63 1)	0 57 1)
100	25	6 25	0 01 1)	0 09 1)	0 02 (1	0 23 1)	0 15 1
100	0	6 25	0 04 1)	0 13 1)	0 05 1)	0 14 1)	0 12 (1)
100	[illegible]	3 12	0 01 (1	0 06 1	0 03 (1	0 26 (1)	0 12 (1
100	0	3 12	0 04 (1	0 06 1	0 06 (1	0 18 (1)	0 07 (1)

Drugs

The f ll ng drugs were used (– Amphetamine bitartrate commercial (+) Amphetamine sulphate (ercial) Desipramine hydrochloride (Pertofran J R Geigy Ltd) Lu 3-010 hydrochl r de H Lundbeck & Co Ltd α Methyl noradrenaline hydrochloride (Corbasil, Hoechst An lin Ltd), Nialamide Niamidal The Swedish Pfizer Ltd Protriptyl ne hydrochloride (Dr C A Stone, Institute for Therapeutic Research) Reserpine (Serpasil The S Ciba Ltd

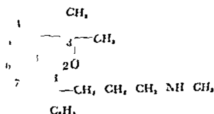
TABLE IV. Effect of various doses of (+)-amphetamine on L-dopa-induced amine accumulation in rat brain and heart

(+)-Amphetamine was given 45 min and L-dopa 30 min before sacrifice. The rats were pretreated with reserpine (10 mg/kg i.p.) and nialamide 22 and 4 hrs before sacrifice respectively. Control rats were treated in the same way as the experimental animals except that no (+)-amphetamine was given.

The values are means ± standard errors of the means, expressed in μg/g tissue. Figures in brackets indicate number of experiments. Each experiment was performed on 2 or 3 pooled organs.

Drugs mg/kg			Heart		Brain		
Nialamide i.p.	(+)-Amphetamine i.p.	L-dopa s.c.	Noradrenaline	Dopamine	Noradrenaline	Dopamine	3-Methoxytyramine
150	7.5	50	0.05 (1)	6.21 (1)	0.06 (1)	2.31 (1	3.02 (1)
150	0	50	0.13 (2) ±0.000	6.23 (2) ±1.210	0.19 (2) ±0.025	3.87 (2) ±0.830	2.05 (2) ±0.170
100	5	25	0.04 (1)	1.68 (1)	0.03 (1)	0.88 (1)	1.16 (1)
100	2.5	25	0.04 (4) ±0.004	1.37 (4) ±0.152	0.08 (4) ±0.021	1.01 (4) ±0.163	1.60 (4) ±0.108
100	0.5	25	0.07 (6) ±0.011	1.53 (5) ±0.309	0.06 (3) ±0.012	1.32 (3) ±0.015	1.23 (3) ±0.104
100	0.25	25	0.05 (2) ±0.015	2.25 (2 ±0.495	0.04 (2) ±0.009	1.17 (2) ±0.070	0.76 (2) ±0.020
100	0.15	25	0.06 (3) ±0.019	2.35 (3 ±0.238		—	—
100	0.10	25	0.08 (3 ±0.024	1.75 (3 ±0.235	0.12 (1)	1.27 (1)	1.08 (1)
100	0	25	0.10 (31) ±0.004	1.87 (24 0.109	0.15 (22) ±0.007	1.24 (21) ±0.048	0.92 (13) ±0.060

Lu 3-010 is a bicyclic phtalane derivative with imipramine-like activity developed by Petersen *et al.* (1966). Its structural formula is



Doses refer to the salts except for amphetamine and α-methyl NA, where they refer to the base. The drugs were generously supplied by the above mentioned companies.

TABLE V Effect of various doses of (+) amphetamine on L-dopa induced catecholamine accumulation in different parts of rat brain

(+) Amphetamine was given 45 min and L-dopa (25 mg/kg s c) 30 min before sacrifice The rats were pretreated with reserpine (10 mg/kg i p) and nialamide (100 mg/kg i p) 22 and 4 hrs before sacrifice, respectively Control rats were treated in the same way as the experimental animals except that no (+) amphetamine was given

The values are means ± standard errors of the means expressed in μg/g tissue Each experiment was performed on pooled tissue parts of 3 rats *Note* The "striatum" contained also adjacent structures rich in NA

(+) Amphetamine mg/kg i p	Noradrenaline			Dopamine			Number of exp
	Stem	Hemispheres	"Striatum"	Stem	Hemispheres	"Striatum"	
0.50	0.15 ±0.019	0.02 ±0.000	0.02 ±0.003	1.18 ±0.172	0.95 ±0.185	1.66 ±0.382	3
0.15	0.31 ±0.073	0.08 ±0.010	0.08 ±0.022	1.26 ±0.093	1.02 ±0.100	1.83 ±0.138	4
0.10	0.26 ±0.022	0.09 ±0.009	0.08 ±0.010	1.31 ±0.082	1.03 ±0.047	1.76 ±0.170	3
0	0.30 ±0.021	0.12 ±0.011	0.10 ±0.008	1.12 ±0.074	0.94 ±0.071	1.74 ±0.124	10

Results

Biochemistry

In rats pretreated with reserpine and nialamide, desipramine in doses down to 2.5—5 mg/kg largely prevented the accumulation of NA in heart following L-dopa administration (Table II) A corresponding effect on brain NA became apparent in a dose of 10 mg/kg Protriptyline showed about the same activity as desipramine in the heart though the possibility of differences in effectiveness between the two drugs cannot be excluded with the present limited material In the brain protriptyline seemed to be more effective than desipramine an effect was already seen with a dose of 5 mg/kg The tendency for both drugs was that the effect in the brain was weaker than in the heart With Lu 3-010 a preventive effect on NA accumulation in heart was seen in the same dose range as for desipramine and protriptyline while no effect was observed in the brain even after the largest dose (25 mg/kg)

In experiments with desipramine 25 mg/kg (to reserpine nialamide pretreated rats) followed by various doses of L-dopa reduced NA levels in brain were observed after all dopa doses employed and in the heart after doses up to 25 mg/kg (Table III) The DA and MT levels were not affected by the desipramine treatment to any significant degree The experiments show that neither the dopa nor the nialamide dose seem to be of critical importance to the results An effect of desipramine was seen at all doses a tendency to weaker effect at the largest dose of dopa ca

TABLE VI Brain monoamine levels after (+) amphetamine (2 5 mg/kg i p) to rats pretreated at different intervals with malamide (100 mg/kg i p)

(+) Amphetamine was given at different intervals after malamide and the animals were sacrificed 1 hr after (+) amphetamine Control rats received only malamide
The values are means ± standard errors of the means expressed in μg/g brain Each experiment was performed on 3 pooled brains

Interval malamide amphetamine	Noradrenaline	Normetanephrine	Dopamine	3 Methoxytyramine	Number of exp
1 hr	0 55 ±0 015	0 02 ±0 007	0 92 ±0 040	0 13 ±0 017	3
Controls	0 57 ±0 023	0 04 ±0 021	0 90 ±0 069	0 09 ±0 004	3
2 hrs	0 55 ±0 031	0 11 ±0 031	0 98 ±0 047	0 18 ±0 029	4
Controls	0 67 ±0 067	0 03 ±0 004	0 96 ±0 070	0 09 ±0 013	5
3 hrs	0 62 ±0 015	0 06 ±0 010	1 10 ±0 157	0 22 ±0 047	3
Controls	0 77 ±0 099	0 04 ±0 000	0 94 ±0 035	0 10 ±0 015	3
4 hrs	0 63 ±0 056	0 09 ±0 015	0 99 ±0 084	0 20 ±0 018	3
Controls	0 69 ±0 006	0 04 ±0 006	0 89 ±0 074	0 08 ±0 007	3

be detected, especially in the heart This is possibly due to the competitive type of inhibition where a weaker inhibition at high substrate concentrations would be expected

Corresponding experiments have been done with various doses of (+) amphetamine (Table IV) An effect on heart and brain NA became apparent already in doses of *0 15—0 25 mg/kg In the higher dose range a decrease in the DA/MT ratio was* also evident, suggesting increased release of DA into the extraneuronal space After 2 5 mg/kg of (+) amphetamine the DA/MT ratio was 0 62 ± 0 066 compared to 1 41 ± 0 165 *in the controls* ($p < 0.025$) Analyses of NM were also performed but most of the values were too low to be regarded as significant with the technique used However, a similar tendency in the NA/NM ratio was found as for DA/MT

Table V shows the effects of (+) amphetamine on L dopa induced NA and DA accumulation in different parts of the brain Doses of (+) amphetamine down to 0 10—0 15 mg/kg partially blocked the NA accumulation in the brain as compared to the controls, the hemispheres being more sensitive to the drug than the brain stem The DA levels in the brain were not affected by the low doses of (+) amphetamine used in these experiments

TABLE VII Brain monoamine levels after various doses of (+) amphetamine to rats pretreated with nialamide

(+) Amphetamine was given 2 hrs after nialamide (100 mg/kg i.p.) and the animals were sacrificed 1 hr later. Control rats received only nialamide.
The values are means ± standard errors of the means, expressed in µg/g brain. Each experiment was performed on 3 pooled brains.

(+) Amphetamine mg/kg i.p.	Noradrenaline	Normetanephrine	Dopamine	3-Methoxytyramine	Number of exp.
5	0.58 ±0.026	0.09 ±0.007	1.02 ±0.033	0.25 ±0.009	3
2.5	0.55 ±0.031	0.11 ±0.031	0.98 ±0.047	0.18 ±0.029	4
1.25	0.67 ±0.028	0.04 ±0.006	1.16 ±0.127	0.14 ±0.008	4
0.5	0.69 ±0.060	0.04 ±0.000	1.05 ±0.065	0.10 ±0.010	2
0	0.67 ±0.067	0.03 ±0.004	0.96 ±0.070	0.09 ±0.013	5

The releasing effect of (+)-amphetamine on endogenously formed amines was also investigated. Rats were pretreated with nialamide at different intervals *before* administration of (+) amphetamine (2.5 mg/kg) and the animals were sacrificed 1 hr afterwards (Table VI). At all intervals studied, except for the shortest interval (1 hr), (+) amphetamine caused a clearcut increase in the levels of 3-O-*methylated* NA and DA metabolites in the brain. NA and DA did not change compared to the controls.

The 2 hr interval between nialamide and (+) amphetamine was chosen to study the effect of various doses of (+) amphetamine (Table VII). Increased levels of NM and MT were possibly observed after 1.25 mg/kg (+) amphetamine and were clearcut after 2.5 mg/kg. The levels of NA possibly tended to be somewhat lower in the higher doses of (+) amphetamine (2.5—5 mg/kg). Otherwise the CA levels seemed to be unaffected.

Histochemistry. In vivo experiments

Reserpine nialamide L-*dopa* (see Table I). After amine depletion by reserpine, nialamide or L-dopa alone did not restore the amine contents of the central CA neurons. However, if L-dopa (10—50 mg/kg) was given to rats pretreated with reserpine and nialamide, a diffuse green fluorescence of weak to strong intensity appeared in the DA rich areas of the brain (neostriatum, nuc. accumbens, and tuberculum olfactorium), in all probability due to an uptake and decarboxylation of L-dopa in the DA nerve terminals of these areas. Furthermore, a large number

(after 10 mg/kg) to strongly (after 50 mg/kg) green fluorescent NA terminals became visible in various parts of the brain, e.g. the nuc. paraventricularis, nuc. periventricularis hypothalami, nuc. motoris dorsalis n. vagi and the neocortex. Also the non-terminal parts of CA axons became distinctly visible — in contrast to normal animals — and varying degrees of increases in intensity were observed in the various groups of CA cell bodies. With the highest dose (50 mg/kg) very marked increases were observed in some groups (the CA cell bodies of the substantia nigra and of the reticular formation of the mesencephalon). The 5-HT neurons were not affected by the dopa treatment and showed the same strong yellow fluorescence intensity as in animals treated with reserpine and nialamide. The pericytes of the capillary wall appeared with a low and high intensity of green fluorescence after a low (10 mg/kg) and high (50 mg/kg) dose of L-dopa, respectively. — An increased background fluorescence was observed with the higher dose, probably due to the presence of dopa in the surrounding brain tissue (cf. Carlsson and Hillarp 1962).

In the higher dose range L-dopa had a clear anti-reserpine effect as judged by the gross behaviour of the animals.

Reserpine-nialamide-desipramine (or protriptyline)-L-dopa. Desipramine or protriptyline largely prevented the accumulation of fluorescence within the NA terminals and non-terminal axons in various parts of the brain. As a rule this effect was most easily detectable with the lower doses of L-dopa used. The NA cell bodies, however, appeared to be unaffected by the antidepressive drugs, probably due to the fact that at this time interval after reserpine treatment (24 hrs) the CA cell bodies have a normal or even higher number of intact amine storage granules (Dahlström and Fuxe 1964). — The peripheral NA terminals appeared less affected by desipramine or protriptyline than the central NA terminals, but a clear reduction in intensity could in many cases be observed, especially with the lower doses.

The DA neurons — cell bodies, non-terminal axons and terminals — were not affected by the imipramine drugs. Neither were the 5-HT neurons affected nor the pericytes.

Reserpine-nialamide-Lu 3-010-L-dopa. The drug Lu 3-010 — in two different doses — did not seem able to prevent to any certain extent the dopa-induced accumulation of fluorescence within the NA neurons (terminal, non-terminal axon and cell body). However, the peripheral NA terminals were affected in the same way as after treatment with the drugs of the imipramine group. The central DA and 5-HT neurons and pericytes were unaffected.

Reserpine-nialamide-(+)-amphetamine-L-dopa. With doses between 3.5 and 7.5 mg/kg of (+)-amphetamine only weakly green fluorescent NA terminals appeared after dopa injection and many did not appear at all; the non-terminal NA axons could in many cases not be seen distinctly. The same was true also for the DA nerve terminals and non-terminal axons. The marked accumulation of fluorescence usually observed in the DA-rich areas was to a large extent prevented and the DA non-terminal axons were indistinct. The DA and NA cell bodies appeared unaffected by the amphetamine treatment, however, probably for the reasons given above.

Thus (+) amphetamine, in contrast to desipramine, affects not only the NA but also the DA neurons The fluorescence intensity of the 5 HT neurons however, were not affected by (+) amphetamine treatment nor was the green fluorescence of the pericytes The peripheral adrenergic terminals appeared in the same way as in the reserpine malamide desipramine-dopa treated rats — With the lower doses (0 15 and 0 5 mg/kg) practically all the various DA and NA terminals appeared as in the controls, except for the NA nerve terminals of the neopallium with the dose of 0 5 mg/kg *With this dose the neocortical NA nerve terminals did not appear at* all or only with a very weak to weak green fluorescence after dopa injection Thus the NA nerve terminals of the neocortex seem to have the highest sensitivity to (+) amphetamine The peripheral adrenergic nerve terminals seemed to be unaffected by this dose

With the higher doses of (+) amphetamine there was a very marked increase in alertness and activity as compared to controls and stereotypic movements (sniffing, gnawing) were observed with a high frequency With the lower doses the psycho motor stimulation still remained whereas the stereotypic activity was hardly observed

Malamide Under perfect reaction conditions it is possible not only to see the very marked increases in the amine levels of the 5 HT neurons after malamide treatment but also to observe a distinct increase in fluorescence intensity in the CA neurons, especially the cell bodies and the non terminal axons and sometimes also in the terminals especially in the fine NA nerve terminals of the cortical areas

Malamide (+) amphetamine After treatment with (+) amphetamine 1 hr before sacrifice, the small but distinct increase usually observed in the CA non terminal axons was no longer present In contrast (+) amphetamine had no effect on the marked increase in the intraneuronal 5 HT levels which occur after MAO inhibition

Histochemistry In vitro experiments

Incubation of brain and vas deferens slices from reserpine pretreated animals with α methyl NA in concentrations from 0 001 to 10 μg ml causes a weak to very strong fluorescence in the CA nerve terminals and non terminal axons (Hamberger and Masuoka 1965) It was suggested that NA is taken up by a mechanism localized to the level of the cell membrane The following CA neuron systems were studied the NA terminals of the neocortex and hypothalamus the non terminal axons in the tegmentum the DA terminals and non terminal axons in the caudate nucleus putamen and finally the peripheral terminals and non terminal axons of the vas deferens

It was found that desipramine from 300 to 3 μg ml strongly inhibited the uptake of α methyl NA added in a concentration of 1 μg ml in the cortex hypothalamus and vas deferens while no significant inhibition of the uptake was found in caudate nucleus putamen If on the other hand an α methyl NA concentration of 0 1 μg ml was used desipramine in as low concentrations as 0 3—0 03 μg ml was able to completely inhibit the uptake in the central and peripheral NA terminals but not in the DA terminals In the sections from the hypothalamic

were sometimes found bundles of strongly fluorescent non terminal axons also after high doses of desipramine 300 µg/ml. These axons probably belonged to DA neurons because uptake by non-terminal axons was never found after desipramine when slices were taken from the tegmentum, caudal to the substantia nigra area.

The *in vitro* experiments made with protriptyline and Lu 3-010 showed that these drugs also strongly inhibited the accumulation of α methyl-NA in the NA terminals and non-terminal axons both in the central nervous system and vas deferens, while no effect could be detected in the DA non terminal axons and terminals. In the experiments where slices were made from animals which had been injected with desipramine or Lu 3 010 before sacrifice, it was found that only desipramine inhibited the uptake by the central NA terminals, whereas both desipramine and Lu 3 010 inhibited the uptake by the NA nerve terminals of the vas deferens.

Experiments with (+)-amphetamine *in vitro* showed that preincubation with this drug for 15 min in a concentration down to 0.2 µg/ml completely inhibited the accumulation of α methyl-NA in both the NA and DA terminals and non terminal axons in the central nervous system. The uptake in vas deferens was completely inhibited. With 0.075 µg/ml a distinct effect could be found only in the thin NA terminals of the neocortex.

Discussion

The injection of L-dopa to reserpine treated animals results in the accumulation of NA — formed via DA — in brain as well as peripheral sympathetically innervated tissues, provided that MAO has been inhibited beforehand. This formation of NA can be detected biochemically. Histochemically the specific fluorescence of the central and peripheral NA fibres is restored by the same treatment, although the intraneuronal distribution is abnormal, owing to blockade of amine uptake by the storage granules (cf. Malmfors 1965). This formation of NA and restoration of fluorescence in NA fibres can be partly prevented centrally as well as peripherally, by desipramine and protriptyline. These drugs are known as potent blockers of the reserpine resistant amine-concentrating mechanism located at the level of the cell membrane of peripheral NA neurons (Malmfors 1965, Hamberger and Masuoka 1965, Carlsson and Waldeck 1965 a, b, c). Furthermore, the *in vitro* experiments on tissue slices show that these drugs block the amine-concentrating mechanism of both central and peripheral NA nerve fibres, irrespective of whether the drugs have been added to the incubation medium or administered beforehand to the animals.

The observations with the compound Lu 3 010 support the view of a close relationship between a) blockade of NA formation from dopa, b) blockade of dopa induced restoration of fluorescence of NA fibres, and c) blockade of the amine-concentrating mechanism. *In vivo*, this compound exerts all three effects on peripheral tissues — a very efficient blockade of ^{3}H NA and ^{3}H metaraminol uptake by heart has been observed by Carlsson and Waldeck (1966 a) — but in the brain effects a) and b) could not be detected. *In vitro*, Lu 3 010 was active with respect to c) both centrally

and peripherally, when added to the incubation medium However, when the agent was injected to the animals before the *in vitro* test, activity was detected in peripheral tissues These data suggest that this agent is an efficient blocker of amine uptake *per se* but is unable to reach the central NA fibres in sufficient concentration *in vivo* At any rate, the data strongly indicate a close connection between actions a), b) and c) The question then arises how these actions are interrelated

In all probability the monoamine synthesizing enzymes are located intraneuronally (Carlsson and Waldeck 1963, Andén, Magnusson and Rosengren 1965) When dopa is administered, it therefore presumably enters the DA and NA nerve fibres and is then decarboxylated to form DA Since the storage mechanism of the amine granules is blocked by the previous reserpine treatment and the MAO is inhibited the DA will accumulate in the extragranular cytoplasm of the nerve fibres Some of the DA will leak out through the cell membrane but will partly be transported back by the amine-concentrating mechanism located at this level When this mechanism is blocked by one of the imipramine like drugs the intraneuronal concentration of DA is reduced Less DA will then be available for the DA β oxidase and less NA will be formed In addition, the net loss of NA from the fibre will be increased by the blockade of recapture This will explain action a) As to action b) the restoration of the fluorescence in NA fibres is probably due to accumulation of both DA and NA for the reasons given above the accumulation of both amines is largely prevented by these agents

It may be argued that the results might as well be explained by an inhibitory action of the imipramine drugs on DA β-oxidase However these drugs seem to be devoid of such activity (Creveling *et al* 1962)

The histochemical *in vitro* and *in vivo* experiments revealed the interesting phenomenon that the ability of the imipramine like drugs to block amine uptake appears to be restricted to NA neurons No effect could be observed on DA neurons whose amine concentrating mechanism must then be assumed to differ from that of NA neurons (As to 5 HT neurons all attempts to reveal a reserpine resistant uptake mechanism have so far been unsuccessful) This may partly explain why the imipramine like agents were unable to influence the over all accumulation of DA in central tissues However other factors probably contribute Under the present experimental conditions strong fluorescence was observed in the pericytes of the capillary wall and this fluorescence was largely unaffected by the imipramine agents *in vivo* It should be noted that even in heart which does not contain DA nerve fibres to any detectable extent nor DA accumulating pericytes the DA accumulation was uninfluenced by these agents even though the restoration of fluorescence in the NA nerve fibres seemed to be partially prevented It may be concluded therefore, that the DA accumulating under the present conditions centrally as well as peripherally is probably to a considerable extent located extraneuronally

Under normal conditions the imipramine drugs do not seem to interfere with N formation The reason for this may be that the concentration of DA in the cy outside the granul_ rmally so low that no appreciable leakage thro

membrane takes place Under the present experimental conditions this concentration is increased by the combination of three procedures i e 1) blockade of storage granules by reserpine, 2) MAO inhibition and 3) loading with precursor

The present investigation revealed both similarities and differences between (+) amphetamine and the imipramine group Both types of agents largely prevented NA accumulation and restoration of fluorescence in NA nerve fibres following dopa administration they also inhibited the amine uptake by NA fibres *in vitro* The most conspicuous difference observed was that (+) amphetamine unlike the imipramine agents is active also on DA neurons *This was immediately evident from* the histochemical *in vitro* and *in vivo* experiments Also the biochemical data showed an effect on DA metabolism which could not be detected after the imipramine drugs the ratio of DA to its O methylated metabolite 3 methoxytyramine was lowered indicating an increased net release of DA into the extraneuronal space

Some further interesting features of (+) amphetamine s interaction with catecholamines should be pointed out *First of all* this drug was found to act with some degree of selectivity on the cortical NA nerve fibres This may be significant in view of the psychomotor activity of this agent *Whether a corresponding selectivity exists* in the case of the imipramine like drugs remains to be investigated Furthermore an effect of amphetamine on catecholamine metabolism could be demonstrated using a rather simple experimental design the accumulation of the O methylated ic metabolites of both DA and NA following MAO inhibition was accelerated by moderate doses of (+) amphetamine An apparently corresponding histochemical effect could be detected on non terminal CA axons where a decrease in fluorescence intensity was observed Preliminary biochemical and histochemical data suggest that the imipramine like drugs are devoid of such activity — In this connection it should be mentioned that tranylcypromine which is a MAO inhibitor with amphetamine like structure and activity has been found to cause a much more marked and rapid increase in normetanephrine levels in mouse and rat brain than nialamide (Carlsson Lindqvist and Magnusson 1959 1960)

The question arises whether the different behavioural effects of the two types of agents may be due to the fact that amphetamine acts on both DA and NA neurons and the imipramine like drugs on NA neurons only This might seem a reasonable suggestion and is not contradicted by the present observations However it must be kept in mind that other differences exist The fact that amphetamine but not the imipramine agents have strong peripheral sympathomimetic actions argue in favour of a difference in NA releasing activity This difference persists after blockade of the uptake mechanism of the granules by reserpine as demonstrated by histochemical as well as biochemical data (Malmfors 1965 Carlsson and Waldeck 1966 b) In fact the amine releasing activity of amphetamine can be detected after low doses which do not seem very active in blocking the uptake mechanism of the cell membrane (cf Carlsson and Waldeck 1966 c) The mechanism of this amine releasing activity which may explain the increase in the O methylated basic DA and NA metabolites observed after amphetamine but not after the imipramine drugs in the present

experiments on animals pretreated with nialamide, remains obscure It may be a question of displacement from hypothetical extragranular binding sites (cf Hu gott *et al* 1963) or, alternatively, of an effect on the cell membrane, leading increased release With respect to the central actions of amphetamine, which are sustained with but little tendency to tachyphylaxis, the latter alternative might seem more likely That the effect of amphetamine is indirect and mediated by endogenous CA is indicated by the fact that its activity is blocked by inhibitors of CA synthesis (see Introduction)

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Adrenergic Nerve Function, Noradrenaline Level and Noradrenaline Uptake in Cat Nictitating Membrane after Reserpine Treatment

By

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Abstract

Andén, N.-E. and M. Henning *Adrenergic nerve function, noradrenaline level and noradrenaline uptake in cat nictitating membrane after reserpine treatment* Acta physiol scand 1966 67 498—504

After reserpine injection to cats (0 1 mg/kg subcutaneously) there was a severe impairment of the adrenergic transmission in the nictitating membranes with recovery after 48 to 72 hrs The noradrenaline of the nictitating membrane was virtually completely lost at the time of the disturbed nerve function and showed no significant restoration at the time of functional recovery The uptake of tritiated noradrenaline by the nictitating membranes was blocked by reserpine but was partially restored after 48 to 72 hrs thus coinciding with functional recovery It is suggested that an intact adrenergic transmission is dependent on the ability of the amine storage granules to incorporate noradrenaline in a small fraction immediately important for the function

Reserpine lowers the content of noradrenaline (NA) in sympathetically innervated organs and blocks the adrenergic transmission from postganglionic sympathetic neurons (Bertler, Carlsson and Rosengren 1956 Carlsson *et al* 1957, Muscholl and Vogt 1958) In addition, reserpine inhibits the tissue uptake of NA from the blood (Muscholl 1960, Hertting, Axelrod and Whitby 1961) It is to be noted that two different mechanisms operate in the amine uptake by neurons one located in the cell membrane and blocked by *e g* cocaine and imipramine but insensitive to reserpine, the other located in the storage granules and blocked by reserpine (Carlsson, Hillarp and Waldeck 1963, Hillarp and Malmfors 1964, Malmfors 1965) In previous publications (Anden, Magnusson and Waldeck 1964, Carlsson 1965) data have been presented indicating that after reserpine treatment no clearcut relation exists between the reduced tissue NA level and the disturbed sympathetic nerve function The nerve function seems to recover at a time when the NA levels still are very low but when the uptake ability is partially restored In the pr[illegible] vestigation an attempt has been made to correlate quantitatively in one

The stimulator was a Grass model S4G. The contractions of both nictitating membranes were recorded semi-isometrically by Grass force displacement transducers model FTO3. The initial tension was set at 2.00 g. Arterial pressure was recorded from a femoral artery by a Statham pressure transducer P23DC.

Following the nerve stimulation ^{3}H-NA[1] *in a dose of 1.00 μg/kg b.w.* was injected into an external [illegible]

counter (Carlsson and Waldeck 1963). Both irides were also analyzed for their content of NA according to Häggendal (1963). The brain, heart and spleen were analyzed for NA and dopamine according to Bertler, Carlsson and Rosengren (1958) and Carlsson and Waldeck (1958), respectively.

Results

The results are presented in Fig. 1. In normal cats sympathetic stimulation resulted in an increase of the tension of the nictitating membrane by 0.26 g (SEM = 0.054, n = 7) at 0.2 imp/sec and by 0.90 g (SEM = 0.047, n = 7) at 20 imp/sec. The contractions were approximately maximal at 20 imp/sec. *At 12—36 hrs after the administration of reserpine* the response of the nictitating membrane to low stimulation frequencies (*e.g.* 0.2 imp/sec) was between 20 and 40 *per cent of the normal*. The response to high stimulation frequencies (*e.g.* 20 imp/sec) showed a less pronounced decrease or to 50—70 per cent of the normal. The minimum response seemed to take place at somewhat different times, it occurred at the lower stimulation frequency after 12 hrs whereas at *the higher one it occurred after 36 hrs*. Normal responses were obtained to stimulation at all frequencies after 48 or 72 hrs, *i.e.* the nerve function recovered completely between 36 and 72 hrs. The unanesthetized animals showed the usual signs of reserpinization, *e.g.* sedation, miosis, ptosis and nictitating membrane relaxation, during the period of lowered stimulation response and all these phenomena disappeared about 48 hrs after the reserpine injection. Reserpine was reinjected in the same dose (0.1 mg/kg s.c.) to 3 cats 48 hrs after the first one when the signs had disappeared. At the examination 24 hrs afterwards all the cats showed about the same impairment of the sympathetic nerve function as the cats without reserpine pretreatment.

The amount of NA in the normal nictitating membrane was 58 ng/pair (SEM = 1.7, n = 13), *i.e.* 0.29 μg/g tissue. The NA in the nictitating membranes decreased to about 10 per cent of the *normal value within 12 hrs after the reserpine* administration. No significant change in the NA level then occurred during the time up to 72 hrs. It should be pointed out that after 72 hrs the response of the nictitating membrane to nerve stimulation *was about normal*. The NA level in the membranes then slowly increased but was only about 40 per cent of the control value as late as 14 days after the administration of reserpine. The NA in the heart and spleen was lowered to about the same extent as in the nictitating membranes (Fig. 2). Nor was there any tendency to rise in the NA content of these organs at 48 *hrs after the* reserpine

[1] Commercially available dl-NA-7-^{3}H HCl with a specific activity of about 7 C/mmole was used. Prior to use the purity was controlled by paper chromatography.

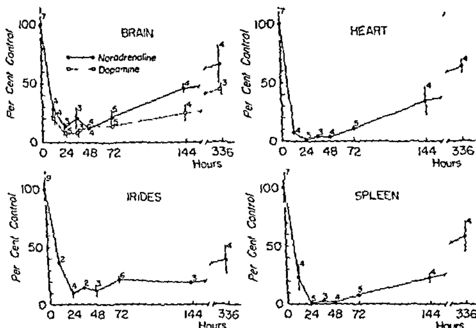


Fig 2 Levels of noradrenaline (means ± SEM, the figures indicate number of experiments) in irides heart spleen and brain and of dopamine in brain at different intervals after reserpine injection to cats (0 1 mg/kg s c) Normal values irides 5 0 μg/g heart 1 1 μg/g spleen 2 2 μg g brain noradrenaline 0 19 μg/g brain dopamine 0 30 μg/g

erpine injection when the nerve function was almost restored After 72 hrs the NA concentration seemed to have increased somewhat which of course was easier to detect in these very NA rich organs In the brain the loss of NA appeared to follow about the same time course as in the peripheral organs As previously found (*Carlsson et al* 1957) *the NA in the brain was not as sensitive to the depleting action* of reserpine as in the sympathetically innervated organs

The ^{3}H NA in the nictitating membrane of normal cats 30 min after an i v injection of ^{3}H-NA in a dose of 1 00 μg kg b w was 0 41 ng/pair (SEM = 0 125, n = 7) i e 2 5 ng g tissue The uptake of ^{3}H NA by the nictitating membranes was almost completely inhibited (less than 10 per cent of the control value) at 12—36 hrs after the reserpine administration After 48 hrs the uptake had increased to 20 per cent of the normal value The uptake then seemed to be rather constant at about 30 per cent for a considerable time The recovery of the ability to take up this quantity of ^{3}H NA seemed to coincide with the return of the nerve stimulation response to the normal The uptake of ^{3}H NA after 72 hrs was significantly ($p < 0 01$) higher than those after 24 and 36 hrs The reappearance of a normal uptake is apparently a slow process since the uptake still after 14 days was depressed by about 50 per cent At no interval was any contraction of the nictitating membranes observed at injection of the ^{3}H NA

Discussion

The results of the present investigation clearly show that there is no correlation between sympathetic nerve function and tissue NA level in the recovery phase after reserpine treatment Thus the data presented here confirm the preliminary ones previously obtained in rats Andén, Magnusson and Waldeck 1964) Nor does the depression of the monoamine levels in the central nervous system accompany the pharmacological effects of reserpine (Häggendal and Lindqvist 1963 1964) The findings may be explained in several different ways *e g* (1) the blockade of the adrenergic transmission after reserpine treatment is independent of the reserpine effect on the NA in the sympathetic nerve terminals (2) the effector cells slowly develop a supersensitivity to NA after reserpine treatment (3) the NA in the sympathetic nerves is stored in pools which have different functional significance and which are depleted for different periods of time after reserpine treatment

The blockade of adrenergic transmission after reserpine treatment is in all likelihood due to a reduced liberation of NA from the sympathetic postganglionic nerve terminals since there is a diminished output of NA to the blood at sympathetic stimulation (Bertler *et al* 1958) and a diminished excretion of NA in the urine (Carlsson *et al* 1957 Gaddum Krivoy and Laverty 1958 Carlsson Boje Rasmussen and Kristjansen 1959) The observations by Bein (1953 that reserpine treatment does not produce ganglionic blockade and does not reduce the response to injected NA are also in agreement with such a view

The recovery of the sympathetic nerve function despite a severe depletion of the NA store could occur at a reduced release of NA if the effector cells had developed a great supersensitivity to NA Actually after reserpine treatment injected NA produces an increased contraction of the nictitating membrane However this effect is seen only after daily administration of reserpine for at least 7 days and does not seem to appear after a single dose of reserpine even if it is large (Fleming and Trendelenburg 1961) We have not observed a contraction of the nictitating membranes to the injected dose of ^3H NA at any interval after the reserpine injection which should have happened if there was a conspicuous supersensitivity Furthermore reinjection of reserpine when the nerve function was restored but when the NA level was still very low produced the same impairment of the transmission as a single injection All these data indicate that a supersensitivity of the nictitating membrane to NA cannot be of major importance for the early recovery of sympathetic nerve function after reserpine treatment

Hillarp was the first to discover that the catecholamines are not homogeneously bound in the storage granules He found two fractions of amines in the granules of the adrenal medulla one large stable fraction bound to an equivalent amount of adenosine phosphates and one small labile fraction bound in an unknown way (Hillarp 1960) Monoamines are taken up *in vitro* by the adrenal medullary granules provided that ATP and magnesium ions are present and this process is efficiently blocked by reserpine in very low concentrations (Carlsson Hillarp and Waldeck

1962, 1963, Kirshner 1962) This incorporation occurs without a cor- upake of ATP, i e the amines are taken up in the ATP free pool Also wh pine is administered to animals the uptake of monoamines by the adrenal med granules is blocked as shown *in vivo* (Bertler, Hillarp and Rosengren 1961, Carl Jonasson and Rosengren 1963) and *in vitro* as well Lundborg 1963) As in the sympathetic nerves the uptake mechanism also in the adrenal medulla recovers much earlier than the amine levels after reserpine treatment, and there seems to be a temporal correlation between the pharmacological effects and the inhibition of uptake (Lundborg 1963, Carlsson, Jonasson and Rosengren 1963 There is, however, one difference between the adrenal medullary cells and the sympathetic nerves concerning the restoration of the uptake function whereas the uptake rapidly increases to the normal in the former case there is only a partial recovery in the latter concomitantly with the return of the nerve function The appearance of this uptake, about 30 per cent of the normal, does not only coincide with the recovery of a normal adrenergic transmission but also with an increase of the excretion of NA in the urine of adrenodemedullated rats to almost control values Henning, unpublished data) The amines taken up in all likelihood occur in the granules since extragranularly located NA does not seem to be released by nerve impulses (Malmfors 1965) Thus, it seems as if an intact adrenergic transmission is dependent on the ability of the storage granules to incorporate NA in a small fraction This fraction may be smaller than the store in which the injected or synthesized amines normally are taken up, even if the amines are preferentially incorporated there This fraction, immediately necessary for the function appears to be so small that it can be detected only with isotope technique (Anden, Magnusson and Waldeck 1964 and the present investigation) or — under certain conditions — with extremely sensitive fluorimetric methods (Haggendal and Lindqvist 1964) Even if the return of the ability to incorporate NA in this labile fraction after reserpine treatment seems to be the most plausible reason for the early recovery of sympathetic nerve function also other factors e g a certain degree of supersensitivity of the effector cells, may contribute

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Microspectrofluorometric Identification of Metaraminol in Sympathetic Adrenergic Neurons

By

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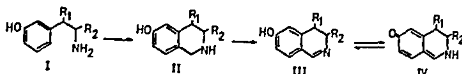
Abstract

JONSSON, G. and M. RITZÉN. *Microspectrofluorometric identification of metaraminol in sympathetic adrenergic neurons.* Acta physiol. scand. 1966. 67. 505—513.

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Primary catecholamines such as dopamine and noradrenaline and certain tryptamines, e.g., 5-hydroxytryptamine are easily converted by formaldehyde gas treatment to intensely fluorescent 6,7-dihydroxy-3,4-dihydroisoquinolines and 6-hydroxy-3,4-dihydro-β-carbolines, respectively in a protein promoted reaction (Corrodi and Hillarp 1963, 1964 and Corrodi and Jonsson 1965a, 1965b). Intermediates in the conversion of both types of substances to their fluorescent compounds are those in a Pictet-Spengler reaction formed 1,2,3,4-tetrahydroisoquinolines and 1,2,3,4-tetrahydro-β-carbolines, respectively. The same reaction also takes place in freeze-dried tissues and can be used for the histochemical demonstration of these amines (for references see review by Hillarp *et al.* 1966).

m-Hydroxyphenylethylamines such as m-tyramine (I, $R_1 = R_2 = H$), m-hydroxyamphetamine (I, $R_1 = H$, $R_2 = CH_3$) and m-hydroxynorephedrine (metaraminol I, $R_1 = OH$, $R_2 = CH_3$) have the molecular requirements for a Pictet-Spengler ring closure to tetrahydroisoquinoline derivatives, namely, by the activating 3-hydroxy group giving an increased electron density at the ring carbo



(III) is in a pH-dependent equilibrium with the tautomeric quinoidal form (IV)

atom 6 where the ring closure takes place (Fig 1) Therefore, it was expected that formaldehyde treated m-hydroxyphenylethylamines would show a fluorescence similar to catecholamines The metahydroxy compounds (m-tyramine, metaraminol, α-methyl-m-tyrosine (α-MMT) and m-tyrosine) enclosed in a dried protein layer exposed to gaseous formaldehyde at + 80° C really exhibit a strong but more yellowish fluorescence than the products from catecholamines The peak of emission being 510 mμ while the fluorescent compunds of catecholamines fluoresce with a maximum at 480 mμ (uncorrected values Corrodi and Jonsson 1966 and Sachs 1965) Metaraminol can be demonstrated by this method in certain cell structures (in cardiac muscle fibers (Sachs 1965), glia cells (Dahlstrom and Fuxe 1964) and pericytes in brain capillaries after incubation of rat cortex slices in a medium containing metaraminol (Hamberger, personal communication)) However, hitherto, it has been impossible to demonstrate this green-yellowish fluorescence intraneuronally after administration of large doses of metaraminol or α methyl m tyrosine with or without reserpine pretreatment (see Sachs 1965), although biochemical and pharmacological investigations have given very strong evidence for the view that these amines are able to accumulate intraneuronally by the very active axon membrane pump (Carlsson and Lindqvist 1962, Andén 1964, Carlsson 1964, Shore *et al* 1964 and Crout and Shore 1964)

A possible explanation for this puzzling phenomenon was obtained during investigations on fluorescent products formed by formaldehyde gas treatment of m-hydroxyphenylethylamines in models (Corrodi and Jonsson 1966) It was found that the fluorescent compounds of m-tyramines, 6 hydroxy-3,4-dihydroisoquinolines, have two emission maxima, one at 510 mμ and the other at 420 mμ (uncorrected values) These findings prompted us to investigate, with a microspectrofluorometric technique (see Caspersson *et al* 1965, 1966), adrenergic neurons after administration of metahydroxy compounds

Material and Methods

used

ent

The following drugs [illegible] (Sigma Chemical Co) DL-α methyl m tyrosine monohydrate [illegible] egis saline) DL-m tyrosine [illegible] (Hoffmann La Roche) ( ) metaraminol bitartrate [illegible] Merck Sharp and Dohme) reserpine (Serpasil ®, [illegible] ved in 0 9 per cent NaCl with the help of a minimal

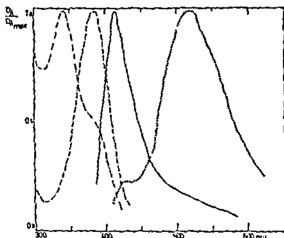


Fig 2 The different types of excitation (— —, — — — —) and emission (———,) spectra of metaraminol in microdroplets treated with formaldehyde gas For explanations see text

amount of dilute HCl, and the solution was then carefully adjusted to a pH of about 5 with sodium hydroxide) All salts were calculated as the free base The i v injections (sublingual vein) were performed under light ether anesthesia The animals were sacrificed by bleeding under ether anesthesia and dissected at once

Superior cervical ganglia and small pieces of ...
gland were taken ra
Then the specimens
demonstration of mo
7 μ and ...

... (se Corrodi et al

Microspectrofluorometry

For the ... fluorometric examinations paraffin ...

Model experiments

m Tyramine hydrochloride (Hoffmann La Roche), 6 hydroxy 3 4 dihydro-isoquinoline hydrobromide (synthesized by Dr H Corrodi see Corrodi and Jonsson 1966) and metaraminol bitartrate (0 5—2 mg salt ml) were dissolved in 2—5 % aqueous solutions of bovine serum albumin or 2 % sucrose containing 0 02 % glycine These solutions were sprayed onto object glasses and dried at room temperature giving protein microdroplets with a diameter of 10—100 μ and a thickness of 0 1—2 0 μ The glasses were exposed to gaseous formaldehyde generated from paraform aldehyde equilibrated with an atmosphere of 30—90 % relative humidity according to Hamberger et al 1964 Excitation and emission spectra were recorded

Results

Model experiments

Analyses of fluorescence spectra of the metahydroxy compounds metaraminol, α methyl m tyrosine m tyramine and m-hydroxyamphetamine) in dried droplets after formaldehyde gas treatment at ...

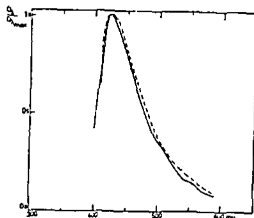


Fig. 3

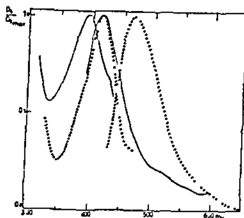


Fig 4

Fig 3 Emission spectra obtained from adrenergic ganglion cells after injection of metaraminol (———) and α methyl m-tyrosine (– – – –), showing spectra characteristic of *metahydroxy* compounds (emission max at 415 mμ)

Fig 4 Excitation and emission spectra obtained from normal adrenergic ganglion cells (   ) showing characteristic catecholamine fluorescence (excitation/emission maxima 415/470 mμ) and after injection of m-tyrosine (———) with nialamide pretreatment (maximal excitation/emission 385/415 mμ)

sion peak at 520 mμ, maximally excited around 385 mμ (see Fig 2) Exposing the droplets to gaseous hydrogen chloride in a closed vessel resulted in a shift of excitation maximum to 345 mμ and peak of emission changed to 425 mμ It was often difficult to produce this shift in emission quantitatively *in protein droplets, probably* due to the buffer capacity of the protein The synthetical 6-hydroxy-3,4-dihydroisoquinoline showed the same spectral variations It was also found that treatment of the m-hydroxyphenylethylamines *with relatively dry formaldehyde* gas resulted in preponderance of the 415 mμ emission peak (see Fig 2)

Tissue sections

Following administration of large doses of metaraminol (2 × 12 mg/kg i p , a 2 hr interval between the doses) and α-methyl-m tyrosine (2 × 400 mg/kg i p , a 12 hr interval between the doses) the intense green fluorescence (seen through a filter with high absorption below 480 mμ) of the adrenergic ganglion cells (Fig 4) due to the presence of noradrenaline almost completely disappeared *within 3—6 hrs after the* second dose (*cf* Norberg 1965) However, a weak greenish fluorescence could be seen even after the largest doses When examining these cells microspectrofluorometrically, a typical emission maximum at 415 mμ was obtained (see Fig 3) Part of this blue fluorescence could also be seen in the microscope with a suitable cut-off filter (e g 50 per cent *transmission at 410 mμ*)

m-Tyrosine (3 × 400 mg/kg i p , at an interval of 2 hrs, sacrificed 2—4 hrs after the last dose) given 2 hrs after injection of nialamide (100 mg/kg i p) resulted in a marked bluish fluorescence of the adrenergic cell bodies with *the spectral properties* excitation around 390 mμ and emission at 415 mμ (see Fig 4) The normal green

fluorescence due to noradrenaline almost completely disappeared (*cf* Nor and no emission peak at 470 mμ could be detected However, a marke of the diffuse background fluorescence, with a maximum of 510 mμ, was

Pretreatment with reserpine (10 mg/kg i p) 12 hrs before injection of metara (12 mg/kg i p) or α MMT (400 mg/kg i p) and sacrifice of the rats 2—4 hrs aft last dose produced a very weak green fluorescence in the adrenergic neurons observed with a 480 mμ cut off filter) and a simultaneous appearance of a char teristic fluorescence spectrum, emission maximum at 420 mμ In contrast the cyt plasm of the neurons in the control animals, treated only with reserpine merely showed a very faint non specific fluorescence

Parallel to the investigations on the nerve cells in the cervical superior ganglion, fluorescence microscopy and microspectrophotometry was performed on adrenergic nerve terminals in the vas deferens and the submaxillary gland When examined under the fluorescence microscope, a bluish fluorescence could be seen in the nerve terminals after administration of metaraminol and α MMT with or without reserpine pretreatment However, due to the difficulties in avoiding disturbing protein fluo rescence, especially that of surrounding muscle fibers, the spectral characteristics of the bluish fluorescence in these tiny structures were difficult to registrate Nevertheless, it was possible to record a characteristic emission peak at 415 mμ in big adrenergic vascular nerve terminals of the submaxillary gland, indicating uptake and accumula tion of the metahydroxy compounds in the nerve terminals

Since it has been reported (Sachs 1965) that heart muscle fibers are able to accu mulate metaraminol two rats were slowly injected with a large dose of this amine (10 mg/kg i v) and immediately sacrificed In this case the muscle fibers exhibited a yellowish fluorescence as reported by Sachs Spectral analyses revealed an emission at about 510 mμ, similar to the fluorescence of metaraminol in models

In rat cervical superior ganglia, groups of small nerve cells usually are found These cells contain a catecholamine, intensely fluorescent at 470 mμ (Norberg *et al* 1966) After injection of large doses of the metahydroxy compounds (metaraminol and α MMT), the spectral characteristics were left unchanged i e pure catechol amine spectra were obtained excitation at 410 mμ and emission at 470 mμ, identical to the spectra of the adrenergic ganglion cells (see Fig 4) These cells have been subjected to a separate histochemical investigation (see Norberg *et al* 1966

Tissue rat mast cells normally containing 5 hydroxytryptamine with excitation maximum around 390 mμ and the peak of emission at 520 mμ were left unaffected by metaraminol but the spectra indicate a slight uptake of α MMT

In order to further establish the specificity of the fluorescence, sodium boro hydride reduction of the fluorescent products of metahydroxy compounds was performed (see Corrodi *et al* 1964 and Corrodi and Jonsson 1966) The bluish fluorescence disappeared upon reduction and could subsequently be regenerated by renewed formaldehyde gas treatment

The fluorescence products formed from the metahydroxy compounds turn to be very sensitive to ultraviolet light causing the fluorescence to fade co

This phenomenon was observed in models as well as in tissue sections and was markedly more pronounced than that of catecholamines

Discussion

During the last years biochemical and pharmacological investigations have shown that administration of metahydroxyphenylethylamines, such as metaraminol, α-MMT and m-tyrosine, cause a marked depletion of the endogenous noradrenaline both in the peripheral adrenergic nerves and in the brain (Carlsson and Lindqvist 1962, Gessa *et al* 1962, Udenfriend and Zaltzman-Nirenberg 1962, Carlsson 1964, Shore *et al* 1964 and Crout and Shore 1964) This depletion is probably brought about by an uptake and accumulation intraneuronally of the metatyramines thereby causing an almost stoichiometrical displacement of the endogenous noradrenaline in the storage granules The action of α-MMT is largely mediated through metaraminol, as α-methylamino acids, e g , α-MMT have been shown to undergo decarboxylation and subsequent β hydroxylation *in vivo* (Carlsson and Lindqvist 1962, Anden 1964 and Shore *et al* 1964)

The depletion of the endogenous noradrenaline by metaraminol and related substances has also been confirmed histochemically with the specific fluorescence method of Falck and Hillarp, visualizing catecholamines directly within the neuron following administration of metaraminol and α MMT, the specific catecholamine fluorescence practically disappeared within 6 hrs (Carlsson *et al* 1962, Dahlstrom and Fuxe 1964, Norberg 1965, and Sachs 1966) This agrees with the findings in the present investigation

However, since the metahydroxy compounds mentioned above have the capacity to react with formaldehyde thereby yielding an isoquinoline derivative, a fluorescence similar to that of catecholamines would be expected This is, indeed the case in dried protein layers where the metahydroxy compounds exhibit a strong green yellow fluorescence upon formaldehyde treatment with an emission peak at 510 mμ (Corrodi and Jonsson 1966) Regarding the biochemical and pharmacological data, it was quite surprising that this fluorescence could not be detected intraneuronally in contrast to the fluorescence caused by the extracellular presence of metaraminol in brain and tongue tissues and that taken up intracellularly by heart muscle fibers (using a cut off filter absorbing strongly below 480 mμ) (see Sachs 1966) In a recent investigation it was shown that m-hydroxyphenylethylamines (I) enclosed in a dried protein layer readily condense with formaldehyde forming 6-hydroxy 1,2,3,4 tetrahydroisoquinolines (II), which are immediately dehydrogenated to their corresponding 3,4-dihydroisoquinolines (III) The latter is in a pH dependent equilibrium with the tautomeric quinoidal form having maximal absorption in a [illegible] (Corrodi [illegible] nd formal- [illegible] dehyde is quite analogous to that between catecholamines and formaldehyde in a protein layer

The results presented in this report clearly show that metraminol and α MMT cause a disappearance of the specific catecholamine fluorescence in adrenergic neurons and a simultaneous appearance of a fluorescence peak at 415 mμ (see Fig 3) With the commonly used yellow secondary filters, this blue fluorescence is over looked, which explains earlier histochemical failures to visualize metatyramines intraneuronally (see Sachs 1965) The microfluorometric data, in tissue sections, agree with the parallelly performed model experiments with pure metaraminol, α MMT and authentic 6 hydroxy 3 4 dihydroisoquinoline in dried protein microdroplets When treating the models with formaldehyde gas of relatively high humidity, the greenish yellow peak at 520 mμ dominates whereas treatment of the models with dry gas results in a principle peak at 415 mμ (Fig 2) The peak at 520 mμ can partially be shifted to 415 mμ with hydrogen chloride treatment of the models (cf Corrodi and Jonsson 1966)

As expected the excitation spectra in models have maxima corresponding to the absorption of the quinoidal (IV) and non quinoidal (III) forms, respectively How ever, when the formaldehyde treated models containing metaraminol were exposed to ammonia or hydrochloride vapours it was observed that the shift between short and long wave emission (415 mμ and 520 mμ respectively, see Fig 2) was not accomplished as readily as that of excitation Thus similar to the situation found in adrenergic neurons (see Fig 4), an excitation spectrum with a main peak at 385 mμ and only a shoulder at 345 mμ could show a practically pure 415 mμ emission It remains to be shown if the observed difference in emission characteristics of meta hydroxy compounds when present in adrenergic nerves and in non nervous cells (see above), is pH-dependent or due to some local intraneuronal factor

The specificity of the blue fluorescence at 415 mμ was further tested with sodium borohydride reduction which reduces 3,4 dihydroisoquinolines and 3 4 dihydro β carbolines (Corrodi *et al* 1964 and Corrodi and Jonsson 1966) Since the blue fluorescence disappeared after $NaBH_4$ treatment and could be regenerated upon renewed formaldehyde gas treatment the emission at 415 mμ is undoubtedly due to a 3 4 dihydroisoquinoline

Considering the spectral data presented $NaBH_4$ reduction and fading of the fluorescence upon ultraviolet light irradiation (see results) the present study shows that metaraminol and α MMT are taken up and accumulated in the adrenergic nerves Furthermore the presented results support the view that noradrenaline depletion by metahydroxy compounds is produced by an exchange between the amines

Although the fluorescent products of metaraminol and α MMT have practically the same spectral properties the emission peak at 415 mμ after injection of α MMT is probably due according to biochemical data (Carlsson and Lindqvist 1962 and Andén 1964) to presence of metaraminol intraneuronally

The results further show that reserpine does not prevent the uptake of the meta hydroxy compounds which is in agreement with the view that the site of reserpine is in the storage mechanism in the amine granules (Carlsson *et*

Dahlstrom *et al* 1965, Hamberger *et al* 1964 and Malmfors 1965), and not b blocking the mechanism for amine uptake localized in the cell membrane of th *entire adrenergic neuron*

As noted by previous investigators (Carlsson *et al* 1962, Dahlstrom and Fuxe 1961 Norberg 1965 and Sachs 1965), it was also observed in this study that even afte administration of the largest doses of metaraminol or *a* MMT, a weak greenisl *fluorescence could be seen This emission could be due to a small residue of noradre* naline, since it is difficult to deplete the noradrenaline stores more than 95 per cen (Anden 1964) and/or due to a small amount of fluorescent metahydroxy compounc in the form giving fluorescence at 520 mμ The latter view is supported by the *finding that injection of a MMT and metaraminol to a rat pretreated with reserpine* also gave this weak greenish fluorescence which could not be seen in animals treated only with reserpine

Injection of m tyrosine (nialamide pretreatment 100 mg/kg i p 2 hrs before in order to prevent inactivation of the amine by monamine oxidase) also showed this bluish fluorescence intraneuronally However, a marked increase of the diffuse background fluorescence was observed probably due to the presence of m tyrosine in the non nervous tissue (cf Carlsson *et al* 1962), giving the yellow fluorescence known from model experiments

The results obtained from cell bodies in cervical superior ganglion and nerve rminals in vas deferens and submaxillary gland were essentially the same Un tunately it was very difficult to registrate spectra of the terminals due to disturbing autofluorescence of the surrounding structures Experiments making attempts to eliminate this drawback of the method are in progress

Following administration of large doses of reserpine, only a slight decrease of the yellow 5 HT fluorescence Caspersson *et al* 1966) was observed in accordance with earlier findings on rat mast cells Moran and Westerholm 1963) and mouse masto cytoma cells in tissue culture Van Orden *et al* 1965) A slight increase *in emission* at 420—460 mμ after injecting *a* MMT suggested that some blue fluorescence was superimposed on the yellow 5 HT fluorescence, indicating an uptake of *a* MMT No such spectral changes were observed after metaraminol injection

Ltd Basle Switzerland (m tyramine hydrochlor de)

The investigation has been supported by research grants from the Swed sh Med cal Research Council (12X 711-01) The Therese and Johan Andersson Memorial Foundation and the Wallen berg Foundation

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From the Department of Pharmacology University of Göteborg Sweden

A Versatile Device for Microscopic Spectrofluorometry

By

GEORG THIEME

Abstract

THIEME, G *A Versatile Device for Microscopic Spectrofluorometry* Acta physiol scand 1966 67 514—520

To facilitate a close study of nerve varicosities cell fractions etc by means of histochemical fluorescence techniques an inexpensive microspectrofluorometer has been constructed A fluorescence microscope with a strong light source is combined with a sensitive photometer and two motor-driven grating monochromators The equipment allows recording of excitation emission and absorption spectra from areas about 1 μ in diameter

During the first approach to histochemical fluorescence techniques for visualizing tissue monoamines Hillarp and coworkers made use of a very uncomplicated microscope equipment (Carlsson *et al* 1961) The material under investigation was activated by the light from a high pressure mercury lamp combined with Schott BG 12 filters, and the emission was studied via a Wratten No 15 cut off filter Fluorescence maxima could be estimated through a hand spectroscope or adequate filters As an aid to verify the identity between tissue components and authentic amines studied in model systems, the equipment soon had to be improved The mercury lamp was replaced by a 1600 watt high pressure xenon lamp with a more continuous spectral distribution, and by means of a prism monochromator (Zeiss M4QII) the relative activating maxima could be visually registered (Falck *et al* 1962)

In the course of this histochemical work our need for a complete analyzing device has increased A couple of microspectrofluorometer systems have recently been published Olson (1960), Loeser and West (1962), Chance and Legallais (1963), Van Orden, Vugman and Giarman (1965) and Caspersson, Lomakka and Rigler (1965) have contributed to the development of sensitive instruments Most of the authors have designed their instruments mainly for emission studies To us, activating as well as emission data are of interest Only the device of Caspersson *et al* has a high applicability to different spectral regions, utilizing a continuous light source and two monochromators These, however, are of the prism type which necessitates the incorporation of complicated construction details if a linear wavelength characteristic is desired

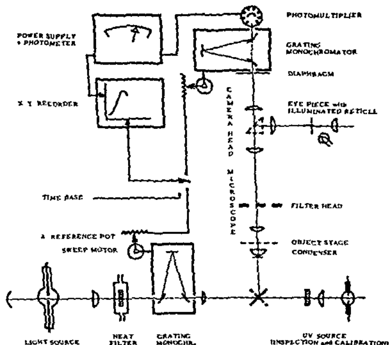


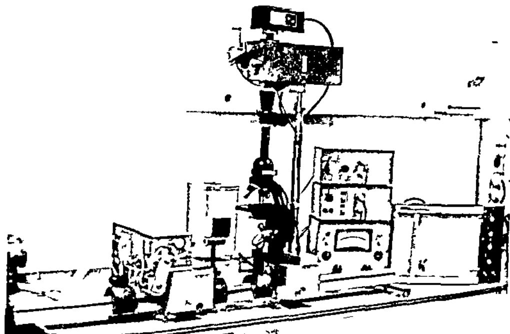
Fig 1 Schematic drawing of the arrangement Components only indicated

To meet our present demands the following facilities should be provided for

1) continuous wavelength ranges of activation and emission,
2) observation and recording of fluorescence from objects sized about 1 μ,
3) adequate sensitivity without the use of too strong activating energies,
4) stability sufficient for quantitative estimations,
5) automatic wavelength scanning combined with chart recording

Construction

With the skilled assistance of Mr Ivan Andersson, instrument maker of this Department a versatile microscopic spectrofluorometer covering these specifications has been constructed Fig 1 indicates the arrangement schematically The light source is a 1600 watt xenon lamp Osram XBO 1600) fed from a special dc power supply Heat radiation is filtered off by a water cooled Schott BG 38/1 before the beam is focussed on the entrance slit of the activating monochromator This one is of the grating type Hilger model 292) with 200—1 000 mμ spectral range aperture f 10 dispersion 7 mμ/mm By means of a reversible synchronous motor and suitable gears a wavelength scanning of 300 mμ is attained in either direction A 3 turn resistor with friction clutch is included in the drive unit Via this voltage divider a variable reference signal is introduced to the wavelength axis of the chart recorder The friction device also allows manual wavelength settings



2 The complete assembly with light shield to the activating monochromator removed Main source to the left with only a part of the condenser visible in the picture

The vertical band of monochromatic light from the exit slit is directed by a surface coated mirror into the dark field condenser (at present type Ultra") of a Zeiss Standard Junior microscope Finally, it is projected on the slide in the form of a narrow strip — For a rapid inspection of large areas it is convenient to turn the mirror against an effective broad band UV source of more suitable dimensions a mercury high pressure lamp (Osram HPK 125 W) with BG 12 filters — The microscope is fitted with a barrier filter holder and, above the ocular, with a camera head The latter includes a retractable prism and an eye-piece with reticle To facilitate observation of the position of the recording area in relation to the fluorescing objects, the eye-piece is provided with a miniature lamp throwing a faint light upon the reticle

The emitted light from the object is focussed in the focal plane of the camera adapter In the center, the area to be analyzed is defined by a diaphragm, acting as entrance aperture to the analyzing monochromator, which is of the same type as the activating monochromator and fitted with an identical drive unit On the exit side a detector is mounted,
to a universal photometer
— 0 001 microamperes for
recorder is transistorized, Moseley model 135 C

As demonstrated in Fig 2 the main components, except the light source, are mounted on an optical bench The two boxes on top of the photometer contain a

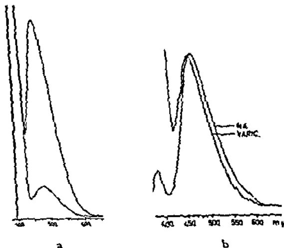


Fig 3 a Emission curve of formaldehyde treated dopamine in serum albumin Lower curve standard serum albumin
Fig. 3 b Comparison of emission curves from a single varicosity (vas deferens nerve mouse) and noradrenaline in a 3 μ layer of protein Formaldehyde treatment

starting unit for the scanning motors, a damping network for the X and Y axis of the recorder, and a time base generator (a variable resistor combined with a synchronous motor, of the same kind as the wavelength references) — The total cost of all components of the complete equipment does not exceed 5,000 US dollars

Performance

The linear wavelength distribution and constant band width of a grating monochromator suits this kind of work very well The activating monochromator might have a higher aperture — it is a question of price — but in combination with a strong light source our arrangement is adequate With clean optical components and a correct set up the stray light will not be of the order of magnitude to interfere seriously with the observations No problems with second-order spectra exist in the practice, as the wavelength ratio activation-emission rarely exceeds 1 1 5

The synchronous type scanning motors give a smooth run and do not influence on the photometer They add an extra facility to the apparatus it is possible to drive the two monochromators simultaneously, fully synchronized With the same wavelength setting of the two monochromators (plus light field condenser) this converts the fluorometer to an absorption spectrophotometer Forward, stop and reverse does not displace the relative settings

The scanning speed has been selected with respect to the band width and the recorder response Five mμ is swept per second with this speed there is little danger of decomposition of the activated object

The size of the recording area is determined by the diaphragm below the emission monochromator (The influence of this aperture upon the band width is trivial in the practical work) With an aperture of 0 6 mm 1/50 of the field of view will be recorded at a spectral band width of less than 5 mμ With maximum magnification power this means a circle about 1 μ in diameter The construction of the mono-

chromator stand allows the use of an extension tube, whereby this area may be further decreased

Sensitivity

It is difficult to find a correct way to specify the sensitivity of this kind of instrument "Minimum detectable light level" does not tell very much The ultimate sensitivity for quinine could be used as a reference, if it did not imply an exact control of the depth of the solution on the slide Absolute volume concentrations are inconvenient to calculate in a fluorescence microscope Fig 3 a gives an example A one per cent protein solution contained 10^{-8} mg/ml of dopamine One microliter was applied on an object glass, dried, and treated with formaldehyde gas The spot was ring shaped, with a surface of 3 5 sq mm, so the thickness was about 3 μ The dopamine concentration was now 10^{-4} mg per ml dry protein, and in the 3 μ layer this resulted in full scale deflection of the emission detector In regard to the low amplifier gain setting during this record it is realistic to count upon a maximal sensitivity at least one hundred times higher As the same deflection will be reached if we use maximum magnification power, this means recording an absolute amount of 10^{-14} μg of the fluorophore, within an area 1 μ in diameter (The lower curve in Fig 3 a reference spot, with no addition of dopamine There is probably a compound in the serum bumin used which reacts in the same way as the test substance The excitation was 410 mμ, i e below maximum to reduce the influence of the scatter No filters were used)

In Fig 3 b the emission from a single varicosity of a vas deferens nerve (mouse) is compared with the emission from noradrenaline in a protein layer (Formaldehyde treatment Excitation 40 mμ below max) No filters were used The record is from a histological standard section To reduce scatter and background fluorescence very thin sections are of great value Plastic embedded slices of 2 μ thickness have given good results according to our preliminary observations

Stability

The Eldorado photometer, with a stability of 0 1 per cent, has worked quite well Evidently, ac amplifiers are not necessary in this work Meter fluctuations sometimes observed have been the result of incorrect positioning of the light beam in relation to the entrance slit of the activating monochromator Appreciable variations in output from the light source have never occurred (otherwise they could easily be compensated for, by means of a monitoring photocell of any kind) However, though the equipment forms a rigid mechanical unit one should avoid manipulations with the emission monochromator during a record The smooth running drive motor does not interfere, but a dislocation of 0 01 mm might cause a 2 per cent change in the deflection

Discussion

Though usually accepted in general laboratory work, the recording technique described is not quite correct The spectral energy distribution curve of the xenon